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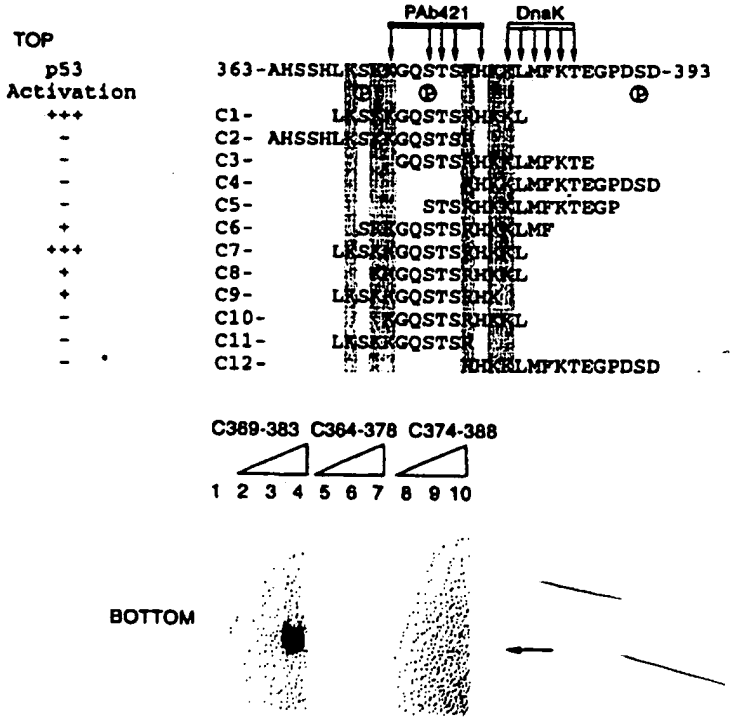
(54) Title: ACTIVATION OF p53 PROTEIN

(57) Abstract

The present invention provides substances which have the property of activating the sequence specific DNA binding activity of latent p53, said substances being based on fragments of the C-terminal regulatory domain of p53 protein, or fragments of murine p53 including the epitope bound by antibody Pab241 (or the corresponding region of human p53). The substances are shown to activate latent p53 and to act synergistically with known p53 activators such as DnaK, monoclonal antibody Pab421 and kinases. The invention includes the use of these substances in methods of medical treatment, and in screening for mimetics and binding partners. A motif of p53 which interacts with the heat shock protein DnaK is also disclosed.



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Activation of p53 Protein

Field of the Invention

The present invention relates to the p53 tumour suppressor protein, and more particularly to the activation of latent p53 protein using substances comprising or based on the C-terminal negative regulatory domain of p53 or the epitope of antibody Pab241. The present invention also relates to the identification of substances based on motifs in the C-terminal domain of p53 that interact with DnaK. The present invention also includes applications of these substances, both therapeutic applications and the use of the substances in screening for mimetics.

Background to the Invention

p53 appears to play a central role in the cellular response to irradiation damage by activating an apoptotic or growth arrest pathway in proliferating cells (Malzman and Czyzyk, 1984; Kastan et al 1992; Kuerbitz et al 1992; Hall et al 1993; Lu and Lane 1993; Zhan et al 1993; Clarke et al 1993; Lowe et al 1993; Merritt et al 1994; Yonish et al 1991).

The precise mechanism by which p53 is activated by cellular stress is of intense interest and may involve both increases in p53 protein level and in p53's specific activity by covalent modification. Although direct activation of the latent sequence-specific DNA binding activity of human p53 can occur through UV and serum-responsive signalling pathways in insect cell systems (Hupp and Lane, 1995), upstream intracellular signalling pathways involved in the direct activation of the biochemical function of p53 in mammalian cells following irradiation have not been clearly delineated nor have the actual enzymes activating p53 function *in vivo* been identified.

Biochemical analysis of p53 has shown that it interacts with many proteins implicated in regulation of p53 protein

function, including; protein kinases and phosphatases, heat shock protein, and DNA binding proteins. The biochemical activity of p53 may also be regulated by interaction of the C-terminus with single stranded RNA or DNA (Oberosler et al 1993; Bakalkin et al 1994; Jayaraman and Prives 1995). The activity of p53 most tightly linked to its tumour suppressor activity is the ability of the protein to bind to DNA sequence-specifically (Kern et al 1992; El-Deiry et al 1992). Inactivating point mutations usually map within the active site for sequence-specific DNA binding or within the central core DNA binding domain (Cho et al 1994; Halazonetis and Kandil 1993; Bargonetti et al 1993). Thus, sequence-specific DNA binding is a biologically relevant function of p53 and understanding its regulation may reveal mechanisms whereby the cell regulates a key damage-responsive pathway.

Biochemical analysis of the regulation of wild type p53 sequence-specific DNA binding has shown that the unphosphorylated tetramer has a cryptic sequence-specific DNA binding activity (Hupp et al 1992). This cryptic or latent state of p53 depends upon a C-terminal negative regulatory domain, which locks the unphosphorylated tetramer in an inactive state. Phosphorylation of the C-terminal negative regulatory domain of latent p53 by either protein kinase C or casein kinase II (Hupp and Lane 1994b; Delphin and Baudier 1994; Takenaka et al 1995), or deletion of this regulatory domain (Hupp et al 1992), activates sequence-specific binding. In addition, a monoclonal antibody or bacterial Hsp70, whose binding sites reside in the C-terminal negative regulatory domain, mimics the effects of protein kinases and activate latent p53 through a concerted transition of sub-units in the tetramer (Hupp and Lane 1994a).

Our earlier patent application WO94/12202 discloses methods of activating mutant p53 including the use of PAb421

antibody or the heat shock protein DnaK, both of which bind to p53 in the C-terminal negative regulatory region.

Summary of the Invention

We have now found that the activation of latent p53 by covalent or non-covalent modification of the C-terminal negative regulatory domain operates in mammalian cells and that the activation of p53 as a sequence-specific transcription factor following UV irradiation does not require increases in protein level and can be mimicked *in vivo* by the intranuclear microinjection of antibody directed to the C-terminal negative regulatory domain of p53. These findings support a model in which each C-terminal negative regulatory domain interacts with a motif in the core of the tetramer and must be displaced to permit the specific DNA binding activity of the protein.

We have also found that small peptides derived from the C-terminal negative regulatory domain can be used to interfere with this intra or intermolecular interaction and activate the DNA binding function of latent p53. Definition of the molecular details of this control pathway that regulates the cellular response to irradiation damage allows the rational design of low molecular weight modifiers of the p53 response. We have also found that the sequence at or around the epitope of antibody Pab241 in murine p53 (or the corresponding region of human p53) is important in activating p53. In addition, we have refined a motif at the C-terminal end of the p53 interacts with the heat shock protein DnaK.

Accordingly, in a first aspect, the present invention provides a substance which has the property of activating the sequence specific DNA binding activity of latent p53, said substance consisting of:

(i) a fragment of the C-terminal regulatory domain of p53 protein, or an active portion or derivative thereof;

(ii) a fragment of murine p53 including the epitope bound by antibody Pab241, or a fragment from the corresponding region of human p53, or an active portion or derivative thereof; or,

(iii) a functional mimetic of (i) or (ii) above;

wherein the fragment or mimetic is optionally coupled to a functional moiety.

In the present invention, "an active portion" means a portion of the p53 peptide which is less than the full amino acid sequence of the fragment above, but which retains the property of activating the DNA binding activity of latent p53.

In the present invention, a "derivative" is a protein modified by varying the amino acid sequence of the protein, e.g. by manipulation of the nucleic acid encoding the protein or by altering the protein itself. Such derivatives of the natural amino acid sequence may involve insertion, addition, deletion or substitution of one or more amino acids, without fundamentally altering the essential activity of the protein.

In the present invention, "functional mimetic" means a substance which may not contain a fragment or active portion of the p53 amino acid sequence, and probably is not a peptide at all, but which has some or all of the properties of the p53 fragment, in particular the property of activating the DNA binding activity of latent p53.

In the present invention, "functional moiety" means a non-p53 derived molecule, for example a label, a drug, or a carrier molecule. In one embodiment, the functional moiety is a carrier molecule is a 16 aa peptide sequence derived

from the homeodomain of *Antennapedia* (e.g. as sold under the name "Penetratin"), which can be coupled to one of the above substances via a terminal Cys residue. The "Penetratin" molecule and its properties are described in WO91/18981. In an alternative embodiment, the functional moiety is a p53 activating molecule. Alternatively, the substances could be coupled to a molecule such as PEG to affect function and prolong the *in vivo* half-life of the substances (see Kuan et al, J. Biol. Chem., 269:7610-7616, (1996)).

Preferably, where the substance is a p53 protein fragment, it comprises a fragment of the C-terminal regulatory domain of p53 protein between amino acid residues 369 and 383. More preferably, the fragment of the C-terminal regulatory domain of p53 includes residues corresponding to the amino acids K370, K372, K373, R379, K381, K382. As demonstrated below, replacement of these residues in the 369-383 p53 fragment reduces the ability of the p53 fragment to activate latent p53.

Alternatively, in embodiments of the invention relating to the epitope of antibody Pab241, preferably the substance consists of fragments of p53 localised between amino acid residues residues 287 to 310 of murine p53, corresponding to residues 293 to 316 of human p53.

In further aspect, the present invention provides a substance which has the property of binding to DnaK, said substance consisting of a fragment of the C-terminal regulatory domain of p53 protein from amino acid residues 381 to 388, or an active portion or derivative thereof.

In a further aspect, the present invention includes composition comprising one or more of the above substances in combination with known p53 activators, e.g. heat shock proteins such as DnaK, antibodies such as Pab421 or Pab241,

or kinases such as casein kinase II. The use of some of these p53 activators is disclosed in our earlier application WO94/12202. Further, modification of p53 by cdc2/cyclin kinase may also interact synergistically with the substances described herein.

In further aspects, the present invention provides pharmaceutical compositions comprising any of the above substances and the use of these compositions in methods of medical treatment. In particular, the present invention relates to the use of these substances in the preparation of medicaments for the treatment of disorders in which the activation of latent p53 is required, such as cancer or other hyperproliferative disorders.

Pharmaceutical compositions according to the present invention, and for use in accordance with the present invention, may comprise, in addition to one of the above substances, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material may depend on the route of administration, e.g. oral, intravenous, cutaneous or subcutaneous, nasal, intramuscular, intraperitoneal routes. For such administration, a parenterally acceptable aqueous solution may be employed which is pyrogen-free and has suitable pH, isotonicity and stability. Those skilled in the art are well able to prepare suitable solutions. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required. Dosage levels can be determined by the those skilled in the art, taking into account the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners. Examples of the techniques and protocols mentioned above

can be found in Remington's Pharmaceutical Sciences, 16th edition, Osol, A. (ed), 1980.

In embodiments in which the substances are proteins, the present invention also provides nucleic acid encoding these proteins. Those skilled in the art can readily construct such nucleic acid sequences from the amino acid sequences disclosed herein, taking account of factors such as codon preference in the host used to express the nucleic acid sequences. In embodiments of the invention in which the protein is coupled to a peptidyl functional moiety, nucleic acid encoding the carrier protein can be linked to the sequence encoding the peptides and the sequences expressed as a fusion.

In further aspects, the present invention provides vectors incorporating the above nucleic acid sequences operably linked to control sequences to direct their expression, and host cells transformed with the vectors.

In a further aspect, the present invention provides the use of any one of the above substances in screening for (i) compounds having one or more of the biological activities of the substances described above or (ii) compounds which are binding partners of one of the substances, e.g. antibodies or complementary peptides specific for the p53 fragments or p53 mimetics, or substances having the same binding properties as DnaK. Conveniently, the candidate compounds can be selected from a synthetic combinatorial library. Examples of screening procedures for mimetics or binding partners include:

(a) immobilising the substances on a solid support and exposing the support to a library of labelled peptides or other candidate compounds, and detecting the binding of the peptides or candidate compound to the substances;

(b) using the substances and a library of unlabelled candidate compound or peptides to find candidate compound

that compete or synergise with the substances in the activation of latent p53 protein;

(c) other combinations of solid phases substrates and binding measurements;

(d) Western blots using the substances and antibodies raised to the substances and determining the displacement of the antibodies by candidate compounds;

(e) using yeast two hybrid screens to detect candidate peptides which bind to the substances or to oligonucleotides derived from the p53 fragments (for a description of yeast two hybrid screens see our earlier application WO96/14334);

(f) using the substances and/or candidate compounds in cell systems to determine whether the fragments or candidate compounds activate latent p53 protein.

In a further aspect, the present invention provides the use of a fragment of p53 including (i) amino acid residues corresponding to K370, K372, K373, R379, K381, and K383 or (ii) amino acid residues comprising the epitope of murine p53 bound by antibody Pab241, or a fragment from the corresponding region of human p53, in the design of an organic compound which is modelled to resemble the three dimensional structure of said amino acid residues, the organic compound having the property of activating latent p53.

In a further aspect, the present invention provides the use of a fragment of p53 including amino acid residues corresponding to 381-388 (KKLMFKT) of p53, in the design of an organic compound which is modelled to resemble the three dimensional structure of said amino acid residues, the organic compound having the property of binding to DnaK.

The designing of mimetics to a known pharmaceutically active compound is a known approach to the development of pharmaceuticals based on a "lead" compound. This might be

desirable where the active compound is difficult or expensive to synthesise or where it is unsuitable for a particular method of administration, eg peptides are unsuitable active agents for oral compositions as they tend to be quickly degraded by proteases in the alimentary canal. Mimetic design, synthesis and testing is generally used to avoid randomly screening large number of molecules for a target property.

There are several steps commonly taken in the design of a mimetic from a compound having a given target property. Firstly, the particular parts of the compound that are critical and/or important in determining the target property are determined. In the case of a peptide, this can be done by systematically varying the amino acid residues in the peptide, eg by substituting each residue in turn. These parts or residues constituting the active region of the compound are known as its "pharmacophore".

Once the pharmacophore has been found, its structure is modelled to according its physical properties, eg stereochemistry, bonding, size and/or charge, using data from a range of sources, eg spectroscopic techniques, X-ray diffraction data and NMR. Computational analysis, similarity mapping (which models the charge and/or volume of a pharmacophore, rather than the bonding between atoms) and other techniques can be used in this modelling process.

In a variant of this approach, the three-dimensional structure of the ligand and its binding partner are modelled. This can be especially useful where the ligand and/or binding partner change conformation on binding, allowing the model to take account of this in the design of the mimetic.

A template molecule is then selected onto which chemical groups which mimic the pharmacophore can be grafted. The

template molecule and the chemical groups grafted on to it can conveniently be selected so that the mimetic is easy to synthesise, is likely to be pharmacologically acceptable, and does not degrade *in vivo*, while retaining the biological activity of the lead compound. The mimetic or mimetics found by this approach can then be screened to see whether they have the target property, or to what extent they exhibit it. Further optimisation or modification can then be carried out to arrive at one or more final mimetics for *in vivo* or clinical testing.

The present invention will now be described by way of example with reference to the accompanying figures.

Brief Description of the Drawings

Figure 1. UV-induced activation of the transcriptional function of p53 does not require an increase in p53 protein levels. (1A) The level of p53 protein in extracts of the irradiated and unirradiated mouse fibroblast reporter cells was measured 16 hours after irradiation at the doses indicated using a two site ELISA assay in which p53 is captured using solid phase monoclonal antibody Pab248 and detected with Rabbit anti-p53 antibody CM5 and an enzyme labelled second antibody. The ELISA was standardized by titration of pure murine p53 protein. (1B) The level of β-galactosidase produced by p53 transcriptional activity was assessed at the single cell level using an immunohistochemical method and the β-gal substrate: (1Bi) 0J/m², (1Bii) 10J/m², (1Biii) 20J/m², (1Biv) 40J/m².

Figure 2. Activation of the sequence-specific transcriptional response of p53 in vivo by microinjection of Pab421. Cells were microinjected with control buffer (2i), the activating antibody Pab421 (2ii), or the inhibiting antibody Pab246 (2iii), incubated for 16 hours, and then stained for β-galactosidase production as in

Figure 1. The complete results of two similar experiments are detailed in Table 1.

Figure 3. (Top panel). Binding sites of activating proteins within the C-terminal regulatory domain of p53. The high affinity amino acid contacts required for Pab421 (Stephen et al 1995) and DnaK binding (data not shown), and the sites of phosphorylation of protein kinase C and casein kinase II are as indicated. The overlap and sequence of synthetic peptides C_1-C_{12}, relative to the carboxy-terminus of p53, are also as indicated. Peptides C_1-C_4 are not biotinylated, while peptides C_5-C_{12} contain an amino terminal biotin group linked to the amino acid sequence SGSG. The shaded amino acids depict high affinity amino acid contacts required for potent p53 activation, which is summarized on the left, from data in Figure 8. (Bottom panel). Incubation of latent p53 tetramers with synthetic peptides derived from the C-terminal regulatory domain. p53 protein (50 nM) was assembled in activation reactions with a panel of synthetic peptides; lane 1 (no peptide); lanes 2-4 (1, 10, and 100 μM peptide C_1); lanes 5-7 (1, 10, and 100 μM peptide C_2); lanes 8-10 (1, 10, and 100 μM peptide C_3). After a 30 minute incubation at 30°C, sequence-specific DNA binding was assayed at 0°C as indicated in Materials and Methods.

Figure 4. (Top panel) Synergistic activation of phospho-p53 with a synthetic peptide derived from the C-terminal regulatory domain. p53 (50 nM) was left unphosphorylated (lanes 1 to 6) or phosphorylated at 30°C with recombinant human casein kinase II ((Hupp et al 1993); 2ng) for 1 minute. After phosphorylation, the reactions were incubated further for 1, 2, 5, and 10 minutes at 30°C. Subsequently, peptide C_1 (20 μM) was added (lanes 6-11) during DNA binding at 0°C and products were analysed as indicated in the Experimental Procedures. -The N-terminal antibody DO-1 was added to a parallel reaction (lane 11 vs

lane 7) to demonstrate p53-specificity and tetrameric nature when bound to DNA. (Middle Panel). Rate of p53 phosphorylation by casein kinase II. p53 (50 nM) was incubated with recombinant human casein kinase II for the indicated time in reactions containing ^{32}P-γ-ATP and phosphate incorporated into p53 was determined as indicated previously (Hupp et al 1992). (Bottom panel). Peptide titration in activation of phospho-p53 tetramers. p53 (50 nM) was phosphorylated with casein kinase II (2 ng) for one minute at 30°C (lanes 1-12) or was left unphosphorylated (lanes 13-15). Subsequently at 0°C either peptide C_1 (lanes 2-5; 5, 10, 20 or 40 μM); potassium chloride (lanes 6-9; 50, 100, 150, and 200 mM); DnaK (lanes 10-12; 1.4, 2.8, or 5.6 μM protein); and peptide C_1 (lanes 13-15; 10, 20, or 40μM) were added and DNA binding was assayed as indicated in Materials and Methods.

Figure 5. Activation of latent p53 with peptide C_1 and DnaK. p53 protein (50 nM) was assembled in activation buffer with increasing amounts of peptide C_1 alone (lanes 1-5; 0 μM, 12 μM, 25 μM, 50 μM, and 100 μM respectively) or with DnaK (2.8 μM) and increasing amounts of peptide C_1 (lanes 6-10; 0 μM, 12 μM, 25 μM, 50μM and 100 μM, respectively) at 0°C for 30 minutes. After the addition of DNA binding buffer and radiolabelled DNA, products were analysed as indicated in Materials and Methods.

Figure 6. Truncation of activated peptide defines endpoints required for high affinity activation. Activation reactions were assembled containing p53 (50 μM), DnaK (2.8 μM), and the indicated synthetic peptides containing truncations (as indicated in Figure 3) at 0°C for 30 minutes. After the addition of DNA binding buffer and radiolabeled DNA, products were analysed as indicated in Materials and Methods.

Figure 7. Alanine substitution of the activating peptide defines essential amino acid contacts. Activation reactions were assembled containing p53 (50 nM), DnaK (2.8 μM), and synthetic peptides (30 μM) containing alanine substitutions at the indicated positions of 1 through 16). Lane 17 represents peptide-16/DnaK-activated p53-DNA complexes shifted by DO-1, demonstrating the tetrameric nature of p53.

Figure 8. Model for peptide activation of latent p53 tetramers. p53 exists stably in the latent state due to interactions between the basic negative regulatory domain amino acid side-chains (shaded cylinders) and a peptide binding pocket within the tetramer. Deletion of the regulatory domain or its phosphorylation and subsequent incubation at high temperature (30°C) disrupts regulatory domain interactions and converts the latent tetramer to an activated tetramer through a concerted transition of subunits. Activation by post-translational modification at the regulatory site can now be separated into two stages.

The first step is a rapid event in which the regulatory domain is modified (ie by covalent modification), but the energy barrier required to disrupt the regulatory domain-tetramer interaction is not overcome. As a result, a stable and latent phospho-intermediate can be isolated. However, in a rate-limiting stage, the activating peptide (darkened cylinder) can effectively compete with the phosphorylated-regulatory domain binding site at 0°C, promoting the dissociation of the phosphorylated carboxy-terminus from its binding site and thus inducing the conformational chance required to activate the tetramer for sequence-specific DNA binding.

Figure 9: Peptides derived from the C-terminal regulatory domain of human p53 compete with DnaK activation. (A) Competitive binding experiments with peptide 379-393 and

all three activating proteins: lane 1 (p53, no activator), lane 2 (p53 + PAb421), lane 3 (p53 + DnaK), lane 4 (p52 + casein kinase II), lanes 5-7 (p53 + PAb421 with 1μg, 0.1μg, and 0.01μg of peptide), lanes 8-10 (p53 + DnaK with 1μg, 0.1μg, and 0.01μg of peptide), and lanes 11-13 (p53 + casein kinase II with 1μg, 0.1μg, and 0.01μg of peptide). (B) Competitive binding experiments using peptide 369-383 which contains the 421 epitope: lanes 1-3 (p53 + PAb421 with 1μg, 0.1μg, and 0.01μg of peptide), lanes 4-6 (p53 + DnaK 1μg, 0.1μg, and 0.01μg of peptide), and lanes 7-9 (p53 + casein kinase II 1μg, 0.1μg, and 0.01μg of peptide). (C) Peptide competition in DnaK-dependent activation reactions. The indicated peptides were incubated in reactions assembled with DnaK and p53 at concentrations of 1μg, 0.1μg, 0.01μg and 0.001μg (lanes 2-5 (peptide 379-393), lanes 6-9 (peptides 374-388), and lanes 10-13 (peptide 369-383)). Lane 1 contains DnaK and p53 incubated at 30°C for 30 minutes. The lack of activation of latent p53 on ice by DnaK (lane 14) demonstrates the temperature-dependence of the activation reaction. After peptide addition and incubation in the reaction, DNA binding was assayed as indicated in Materials and Methods. The position of the p53-DNA complexes are indicated.

Figure 10: Binding of DnaK to human p53 peptides. Biotinylated peptides from the C terminus of human p53 (0.05, 0.5, 5μM) were incubated with DnaK (1μg/assay, 1.5μM final concentration) at 30°C for 30 minutes and then separated on a native polyacrylamide gel. Bound protein-peptide complexes were detected with Streptavidin-peroxidase and ECL.

Figure 11: Binding of human Hsc70 to human p53 synthetic peptides. 1.5μM human Hsc70 was incubated with 0.05, 0.5 and 5μM of the respective C-terminal biotinylated peptides of human p53. Protein bound to the peptides was detected with Streptavidin-peroxidase and ECL following a native gel

electrophoresis and blot onto nitrocellulose. DnaK (1.5μM) incubated with equivalent amounts of peptide 376-390 were run on the same gel as an internal standard for the strength of the interaction.

Figure 12: Binding of DnaK to murine p53 peptides. The binding of 1.5μM DnaK to biotinylated peptides of the C-terminus of murine p53 was established using titrations of the relevant peptides (0.05, 0.5, 5μM). Protein-peptide complexes were resolved by native gel electrophoresis and detected with Streptavidin-peroxidase and ECL. The human peptide 376-390 was run as a control for the strength of the signal.

Figure 13: Activation of murine p53 by DnaK. Latent murine p53 (150ng) produced in bacteria was activated with the monoclonal antibody PAb421 or co-incubated for 30 minutes at 30°C with increasing concentrations (0.05, 0.1, 0.5, 3, 6μM) of the bacterial heat shock protein DnaK prior to analysis for DNA binding by gel electrophoresis at 0°C.

Figure 14: A. Activation of murine p53 by DnaK is dependent on the phosphorylation state of p53. 150ng of wild-type murine p53 produced in insect cells was incubated for 30 minutes at 30°C with increasing concentrations of DnaK (0.05, 0.1, 0.5, 1.5, 3, 6μM) before analysis for sequence-specific DNA binding. In comparison, the 309Ser>Ala mutant produced in insect cells was also subjected to the same treatment. The activation of both proteins with the monoclonal antibody PAb421 is run as a control for maximal activation. B. The monoclonal antibody PAb421 can activate p53 for DNA binding. Inclusion of 200ng of the monoclonal antibody PAb241 (recognizing aa288-297) activates latent, murine p53 produced in insect cells for DNA binding to about 40% of activation by PAb521 (aa366-375).

Figure 15: Mechanism of activation of p53 by DnaK. The DnaK binding site was defined to aa381-388 and human Hsc70 binds to the same site on p53. (The sequence of murine p53 was obtained from the Swiss protein database and it is assumed that the second ATG is used as the initiation codon, which predicts a 387-amino acid protein). Within the DnaK binding site on human p53, there are four amino acid changes in murine p53 which reduce the binding affinity of DnaK and lower the activation extent of murine p53 by DnaK. However, murine p53 is efficiently activated by DnaK if it is assembled and phosphorylated in recombinant insect cell expression systems. One site of phosphorylation that may act in concert (positive cooperation) with activators binding to regulatory domain I, is the cdc2 phosphorylation site (aa309). Consistent with the regulatory role played by this second domain, the monoclonal antibody PAb241 also activates p52 protein for DNA binding.

Figure 16 shows the sequences of the regulatory domains of p53 identified in this application, in addition showing the DnaK binding site.

Table 2: Interaction of Hsp70 isoforms with C-terminal peptides of p53. Protein sequences were obtained from the Swiss Protein data base and analysed for sequence homology with DnaK using the GCG software. Hsc70, sharing the least overall homology with DnaK, interacts strongly with p53 at the same site as DnaK. Hsp70 binds very weakly to peptide 376-390. No interaction could be demonstrated for p53 peptides and the Grp78 (BiP) heat shock protein which was expressed in *E. coli* as recombinant hamster Grp78.

Materials and Methods

Reagents, enzymes and proteins.

Recombinant latent forms of human p53, casein kinase II from rabbit muscle, DnaK, and monoclonal antibodies DO-1, Pab421-Fab fragments, Pab421 were purified as described (Hupp and Lane 1994a; Hupp et al 1992). Assembly of activation reactions, sequence-specific DNA binding reactions, ELISA, DnaK and p53 peptide-binding reactions, and phosphorylations were performed as indicated in the Figure legends. Recombinant human casein kinase II was obtained from Boehringer Mannheim. Synthetic peptides were obtained from Pfizer and Chiron Mimitopes. Human Hsp70 and Hsc70 were expressed in *Escherichia coli* and purified as published elsewhere (Freeman et al). Recombinant hamster Grp78 expressed in *Escherichia coli* and was obtained from StressGen Corp. Non-biotinylated peptides were obtained from Pfizer.

Purification of DnaK.

The cells of a DnaK over-expressing strain were grown shaking in LB media at 30°C to an O.D.(600nm) of 0.5, when they were quickly heat-shocked by two fold dilution in prewarmed (55°C) LB media. Subsequently, the incubation was continued for another 3 hours at 42°C. Cells were pelleted by centrifugation and resuspended in 10% sucrose/50mM HEPES, pH7.6 to an O.D. equivalent to 150. The cell suspension was lysed for 45 minutes on ice by adding KCl to 0.25M, DTT to 2mM, lysozyme to 0.5 mg/ml, benzamidine to 1mM and leupeptin to 1μm filter and then loaded onto a Q50-Sepharose column at a protein(mg):resin(ml) ratio of 10:1. Bound protein was eluted using a linear gradient from 0.05 to 1M KCl (in buffer B, containing 10% glycerol, 20mM HEPES (pH 7.5), 5 mM DTT, and 0.1mM EDTA). Fractions containing DnaK were dialysed against a buffer containing 10% sucrose, 20mM Imidazole (pH 7.0), 20mM $MgCl_2$, 20mM KCl, 5mM DTT, 1mM Benzamidine and leupeptin to 1μg/ml (buffer A). Dialysed

protein was applied to an ATP-agarose column (Sigma A-2767) in buffer A at a protein(mg):resin(ml) ratio of 20:1. After washing in buffer A containing 1M KCl and equilibration in buffer A, DnaK was eluted in buffer A containing 10mM ATP.

Purification of latent human p53 from *Escherichia coli*.

Recombinant human p53 was expressed in BL21 *E. coli* cells at room temperature using a T7 expression system (Midgley et al, 1992), in which latent p53 tetramers were purified from soluble lysates (Hupp et al, 1992). p53 was purified by a modification of a published protocol (Hupp et al 1994a). Briefly, human p53 was purified using Heparin-Sepharose (Hi-Trap, Pharmacia Biotech Inc.), Phosphocellulose (P-11, Whatmann), and Superose-12 (Pharmacia) column chromatography.

Purification of murine p53 protein.

Murine wild-type p53 was expressed in *E. coli* BL21 cells as described for human p53 (Hupp et al 1994a). Murine wild-type p53 and the point mutant Ala309 were also expressed in *Spodoptera frugiperda* cells (Sf9 cells) as described elsewhere (Hansen et al). The protein was purified using Heparin-Sepharose (Hi-Trap), Pharmacia Biotech Inc.) with a linear KCL gradient from 0.1 to 1M KCl in buffer B.

Activation of p53.

p53 (indicated amounts) was added to 10μl of Activation buffer (10% glycerol, 1.0 mg/ml BSA, 0.05 M KCl, 0.1 mM EDTA, 5 mM DTT, 0.05% Triton X-100, 10 mM $MgCl_2$, 0.5 mM ATP (or 50μM ATP when activated using protein kinases) and 25 mM HEPES (pH 7.6)), followed by incubation at 30°C for 30 minutes with indicated activating factor or peptide. Reactions were placed at 0°C at 10μl of a DNA binding buffer (20% glycerol, 1.0 mg/ml BSA, 0.05 M KCl, 0.1 mM EDTA, 5 mM DTT, 0.05% Triton X-100, 10 mM $MgCl_2$, 0.5 mM ATP, 5 ng of radiolabeled consensus site oligonucleotide (Hupp

et al 1992), and 100 ng of supercoiled pBluescript competitor DNA. Reaction products were processed using native gel electrophoresis as indicated previously (Hupp et al 1992).

DNA transfections and UV treatment.

10μg of RGCΔFos LacZ plasmid DNA and 1μg of DOR neo2 plasmid DNA were cotransfected into 5 x 10^5 cells (β-gal cells using calcium phosphate precipitation followed by a 15% glycerol shock. The transfected cells were cultured in selection medium, supplemented with 1μg/ml G418 (β-gal cells) to select cells containing neo plasmid. 14 resistant cells were cloned by cloning ring and expanded. The β-galactosidase activity was measured 12 hours after the cells were exposed to 10 J/m^2 of UV to induce the endogenous wild type p53 and the mot responsive clone was used to carry out further study. When cells were grown to 80% confluence, in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1μg/ml G418, culture medium was removed and the cells were exposed to UV in a XL-1500UV cross-linker (Spectronics Corporation). The energy of UV light delivered was precisely controlled by the cross-linker. After exposure the cells were grown in fresh culture medium for 16 hours before LacZ expression was determined. Cells were then washed with phosphate buffered saline (PBS) and fixed with 2% formaldehyde/0.2% glutaraldehyde in PBS for 5 minutes on ice. Cells were washed with PBS and overlaid with 0.25 mg/ml β-gal in reaction mix (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM magnesium chloride in PBS). Cells were incubated at 37°C for 16 hours and β-gal positive cell numbers determined.

Murine p53 ELISA assay.

Levels of murine p53 were determined in a two site immunoassay as follows. Falcon microtitre dish wells were coated with 50 ml of purified mouse monoclonal antibody Pab

2487 at 30 mg/ml in carbonate coating buffer for 8 h at 4°C. The capture antibody was then removed and the plates blocked with 200 ml 3% BSA in PBS overnight at 4°C. The plates were washed (1 X PBDS, 2 X 0.1% NP-40 in PBS, 1 X PBS) and 50 ml of serially two fold diluted cell extract added for 3 hours at 4°C. Cell extracts were prepared by lysis in ice-cold NET buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% NP-40) containing 2 mM phenylmethylsulphonyl fluoride, for 30 minutes at 4°C. Debris was removed from the lysate by centrifugation at 14000 rpm in a refrigerated Eppendorf centrifuge. Cell lysates were removed avoiding cross contamination and the plates washed as before. Captured murine p53 was detected using 50 ml of the rabbit anti-p53 serum CM5 diluted 1:1000 in 1% BSA in PBS for 2 h at 4°C. The plates were washed and 50 ml horse radish peroxidase conjugated swine anti-rabbit IgG diluted 1:1000 in 1% BSA in PBS was added for 2 h at 4°C. Following washing, bound p53 was visualized with 50 ml TMB substrated per well. The colour reaction was stopped by the addition of 50 ml 1 M H_2SO_4 per well and the optical density at 450 nm measured. The murine p53 ELISA assay was standardized by including known quantities of recombinant murine p53 expressed in *E. coli* and resolubilised from inclusion bodies.

Microinjection.

24 hours prior to microinjection the cells were trypsinised and approximately 200 cells were spotted as 5 ml drops onto tissue culture dishes. Cells were allowed to adhere for 1 hour before culture medium was added. Microinjection was performed using an Eppendorf microinjection system (Microinjector 5242, Micromanipulator 5170) mounted to an Axiovert 35 M with heated stage. Injections were intranuclear. Purified mouse monoclonal antibodies Pab 421 and Pab 246 were injected into a microinjection buffer (100 mM glutamic acid, 140 mM KOH, 1 mM MgS4, pH7.2) at a concentration of 2 mg/ml. Following microinjection, fresh

medium was added to the cell cultures and they were incubated 24 h before being fixed and assayed for LacZ expression with β-gal.

Peptide binding assay.

Peptides used in this assay were purified and obtained from Chiron Mimotopes and contain a biotin group at the N-terminus linked to the amino acid sequence SGSG. Peptides are 15-mers representing the linear amino acid sequence of human or murine p53. DnaK (1.5μM) was incubated with 0.05, 0.5 and 5μM peptide in reaction buffer (15% glycerol, 25mM HEPES, pH 7.5, 50 mM KCl, 5mM DTT, 0.02% Triton X-100, 10 mM $MgCl_2$, 1mM ATP) for 30 minutes at 30°C. Reactions were run on a native 8% polyacrylamide gel at 50V for 3 hours, then blotted onto nitrocellulose as described (30) and the peptides were detected with Streptavidin-conjugated Peroxidase (Sigma) and ECL (Amersham).

Phosphorylation of latent p53.

p53 protein (12ng) was incubated in 10μl of a buffer (10% glycerol, 10mM $MgCl_2$, 20mM HEPES, pH 7.6, 0.1mM ATP, 0.1mM EDTA, 5mM DTT, 0.1% Triton X-100) with casein kinase II from rabbit muscle (Hupp et al, 1993) or recombinant human casein kinase II obtained from Boehringer Mannheim (0.1mU). Incubations were performed for the indicated times at 30°C and reactions were added to 10μl of DNA binding buffer containing radioactive target DNA as described below.

DNA binding assay.

Binding conditions are described in detail elsewhere (Hupp et al, 1992). Briefly, the DNA binding buffer contained 20% (v/v) glycerol, 50mM KCl, 40mM HEPES (pH 7.5), 0.05mM EDTA, 5mM DTT, 0.1% Triton X-100, 10mM $MgCl_2$, and 1.0mg/ml bovine serum albumin. A double-stranded oligonucleotide representing the specific p53 consensus site (PG) (El Deiry et al, 1992, Hupp et al, 1992) was end-labelled with [λ^{32}-P]ATP and used with a 20-fold excess of supercoiled non-

specific, non-labelled competitor DNA (pBluescript II SK+, Stratagene). Incubations of p53 with DnaK or peptide were performed for 30 minutes at 30°C in 10µl reaction buffer. Then 10µl DNA binding buffer containing 5ng labelled PG and 100ng pBluescript were added to the reaction mix, incubated on ice for 30 minutes, loaded on a 4% native polyacrylamide gel and run at temperatures from 4°C to 12°C. For the quantification of the radioactive signals a PhosphorImager (Molecular Dynamics) was used.

Detailed Description

Results

UV induced activation of the transcriptional function of p53 does not require an increase in p53 protein levels: support for a model of p53 activation by postranslational modification.

The levels of p53 protein rise dramatically in some cells and tissues exposed to DNA damaging radiation (Maltzman and Czyzyk 1984; Kastan et al 1992; Hall et al 1993; Midgley et al 1995). This rise in p53 protein following irradiation is associated with enhanced transcription of p53 responsive genes (Lu and Lane 1993; Zhan et al 1993). Although the mechanism by which p53 levels increase in the environment of the irradiated cell is not known, the increase in p53 protein concentration provides a simple explanation for the increase in transcription from p53 responsive genes. However, an alternate mechanism for the regulation of p53 dependent transcription has been suggested by a series of biochemical studies on the regulation of the sequence-specific DNA binding function of p53. These studies show that p53 can be produced in a biochemically latent state that can be activated for specific DNA binding after the modification of a C-terminal negative regulatory domain by distinct enzymes in vitro (Hupp and Lane 1994b).

Furthermore, signalling pathways that respond to UV radiation or growth factors in serum can directly activate p53's sequence-specific DNA binding function in cells (Hupp and Lane 1995). According to this model then, covalent modification of p53 without increase in protein level may be sufficient to activate the p53 response following DNA damage in normal cells.

To test these two models we used a wild-type p53 containing murine fibroblast cell line with a stably integrated p53-responsive β-galactosidase reporter gene. When these cells are exposed to intermediate doses of UV light (20 and 40 J/m^2) two events are observed: p53 levels rise as judged by a sensitive ELISA assay (Figure 1A) and expression of the β-galactosidase reporter gene is activated as determined by cell staining (Figure 1B). Notably, the dose response curve shows that at lower doses of UV irradiation ($10J/m^2$), p53 dependent transcription is activated without any detectable increase in p53 protein level (Figure 1A and 1B). These results imply that the specific activity of p53 as a transcription factor can be altered by irradiation without increasing p53 protein concentrations. In addition, these results suggest that unirradiated fibroblasts contain a pool of latent p53 which can be activated by a factor in response to DNA damage. To establish the existence of this pool of latent p53 in unirradiated cells we sought to activate it independent of DNA damage. The antibody Pab421 is a potent activator of the DNA binding function of latent p53 in vitro, so we tested the capacity of the Pab421 antibody to activate p53 dependent transcription in vivo by microinjection.

Activation of the sequence-specific transcriptional response of p53 in vivo by microinjection of Pab421 establishes the existence of a pool of latent p53 in mammalian cells.

Strikingly, microinjection of the antibody Pab421 activates the p53 transcriptional response, as shown by the production of β-galactosidase enzyme in the injected reporter cells (Figure 2ii). Buffer alone (Figure 2i) or inhibitory antibody, Pab246 (Halazonetis and Kandil 1993), does not give rise to a transcriptional response (Figure 2iii). A quantitative summary of two independent experiments is shown in Table 1. These results reflect an important concept; that a latent pool of p53 can be activated in vivo in the absence of irradiation damage by a post-translational event catalysed by a specific agent targeting the carboxy-terminal negative regulatory domain. Furthermore, they provide an explanation of an earlier paradox in the field. While the viability of p53 null mice establish unequivocally that p53 function is not required for the cell cycle, microinjection of anti-p53 antibodies into the nuclei of quiescent cells has been shown to block entry of these cells into S phase following serum stimulation (Mercer et al 1982; Mercer et al 1984; Deppert et al 1990). These results had been interpreted previously to show that p53 was required for entry into S, as it was reasonably assumed at the time that the antibodies acted as antagonists blocking an essential p53 function required for G1 exit. Our results, based on our in vitro and in vivo studies, clearly demonstrate that these antibodies can instead act as agonists, activating the transcriptional activity of latent p53 in vivo and would therefore be expected to induce a p53 dependent G1 block in the same way as ionizing irradiation. Given the relevance of this activation reaction in vivo, we have focused on determining the mechanism of p53 activation with a view towards

identifying small molecules that can be used to regulate the p53 pathway.

A synthetic peptide derived from the C-terminal regulatory domain activates latent p53

Our current model of how the C-terminal negative regulatory domain of p53 acts to control the DNA binding function of p53 is illustrated in Figure 8. Negative regulation of p53 may occur through amino acid side-chain interactions between the regulatory site and another domain within the tetramer. According to this allosteric model, competitive disruption of the regulatory site amino acid contacts by synthetic peptides derived from the C-terminus would lead to the conformational changes which activate p53. In contrast, according to a steric model of negative regulation, small peptide mimetics of the C-terminus would actually bind to the active site and inhibit sequence-specific DNA binding of p53. To discriminate between these two possibilities, we sought to determine whether p53 function would be inhibited or activated by small peptides derived from its negative regulatory domain.

A panel of small molecular weight, overlapping polypeptides derived from the C-terminal regulatory domain were used for these studies (Figure 3, top panel). Peptide C_1 (C369-383), which harbors both the protein kinase C site and Pab421 epitope, activates p53 at peptide concentrations of 100μM (Figure 3, bottom panel, lanes 2-4 vs lane 1). Synthetic peptides C_2 (C364-378, lanes 5-7), C_3 (C374-388, lanes 8-10) or C_4 (data not shown) at concentrations up to 100μM do not activate p53 function, indicating that activation by peptide C_1 is highly specific. These results are consistent with the model whereby specific amino acid contacts between the regulatory site and a surface sub-domain can be disrupted to give rise to the allosteric activation of p53.

Synergistic activation of latent p53 using casein kinase II and a synthetic peptide derived from the negative regulatory domain.

The activation of the latent sequence specific DNA binding function of p53 seen with peptide C_1 is modest. We sought to find conditions where the effect was enhanced so that we could examine the specificity of the activating peptides in more detail. Specifically, we have taken advantage of the fact that, under specific conditions, stoichiometric phosphorylation of wild type p53 can be uncoupled from its activation (see below, Figure 8).

The rate of p53 activation by casein kinase II was analysed using catalytic levels of recombinant casein kinase II (10-fold lower molar equivalents); under these reaction conditions p53 becomes progressively more active from 2-10 minutes at 30°C (Figure 4, top panel, lanes 1-5), despite the fact that stoichiometric phosphorylation has occurred within 30 seconds (Figure 4, middle panel).

These results indicate that phosphorylation by recombinant casein kinase II is a very rapid step and that subsequent conformational changes leading to p53 activation are rate-limiting.

Under these conditions in which there is lower level of p53 activation, but stoichiometric phosphorylation of the protein, the subsequent addition of peptide C_1 at 0°C dramatically stimulated p53 sequence-specific DNA binding (Figure 4, top panel, lanes 7-11) to achieve a specific activity similar to Pab421-activated p53 (data not shown). The N-terminal antibody DO-1 shifts the migration of this protein-DNA complex (lane 11 vs lane 7) demonstrating the p53-specificity of the reaction. In addition, the tetrameric nature of this peptide-activated form of p53 (lane 11) is clear from the expression of four DO-1

epitopes bound by two DO-1 monoclonal antibodies during native gel electrophoresis, as described previously (Hupp and Lane 1994a).

Alterations in ionic strength (Figure 4, bottom panel, lanes 6-9 vs lane 1) or addition of bacterial Hsp70 (lanes 10-12 vs lane 1) could not lead to a stimulation of sequence-specific DNA binding of latent, phospho-p53 intermediate complexes. Under these same conditions, a prior, rapid and stoichiometric phosphorylation of p53 was essential to unmask the highest level of stimulation of sequence-specific DNA binding by the synthetic peptide (lanes 2-5 vs lanes 13-15).

Synergistic activation of latent p53 using DnaK and a synthetic peptide derived from the negative regulatory domain.

To gain further insight into the activation of p53 by synthetic peptides, we investigated whether a prior modification of the carboxy-terminal regulatory site of p53 by a non-kinase activator could also give rise to synergistic activation by peptides. This second assay takes advantage of the fact that (i) the *E. coli* Hsp70 (DnaK) can also interact with latent p53 tetramers to efficiently unmask the cryptic sequence-specific DNA binding activity *in vitro* (Hupp et al 1992) Hupp et al 1993), (ii) DnaK targets the C-terminal negative regulatory domain at a site within peptide C_3 (data not shown) and (iii) activation of p53 by DnaK can now be divided into two stages; a latent-DnaK-p53 intermediate complex can be stably isolated at 0°C (see below).

Activation of latent p53 by DnaK requires an incubation at 30°C, as activation of p53 does not occur when reactions are incubated at 0°C (Figure 5, lane 6). As DnaK does not activate p53 at 0°C, the ability of peptide C_1 to synergise

in concert with DnaK under these conditions was examined. Importantly, and in contrast to peptide C_3, the activating peptide C_1 does not bind to DnaK (data not shown). As seen previously, a titration of peptide C_1, in the absence of DnaK at 0°C, gives rise to a modest activation of latent p53 (Figure 5, lanes 1-5). In contrast, a titration of peptide C_1, in the presence of DnaK at 0°C (Figure 5, lanes 7-10 vs lane 6), gives rise to a dramatic activation of latent p53 with an apparent K_m of peptide activation being 32μM. The specific activity of DnaK/peptide-activated p53 at 0°C was identical to DnaK-activated p53 at 30°C (data not shown). Alterations in the pH, ionic strength or time of incubation does not lead to a DnaK-dependent-activation of p53 at 0°C in the absence of peptide (data not shown).

Alanine scan of the activating peptide defines specific amino acids required to activate latent forms of p53.

Truncation of the activating peptide defines endpoints required for activation of p53. A panel of biotinylated peptides which contain N-terminal or C-terminal deletions of the activating peptide C_7 (a biotinylated version of the activating peptide C_1, Figure 3), were used to define the end limits required for peptide activation with DnaK at 0°C. A titration of peptide C_7 activates p53 using DnaK with an apparent K_m of activation being 9μM (Figure 6), which is approximately four-fold lower than that obtained with the non-biotinylated peptide C_1 in the presence of DnaK. Truncated derivatives of the full length peptide, peptide C8 or C9, could also co-activate with DnaK but the apparent K_m of activation being slightly higher at 30μM. However, further truncation from the N-terminus (peptide C_{10} or C_{12}) or from the C-terminus (peptide C) prevented peptide activation, thus defining amino and carboxy-terminal endpoints required for a high affinity activation of p53.

A series of peptides with single alanine substitutions at every position from amino acids 369-383 was used to define critical amino acids within the full length peptide which function in the activation of p53 (Figure 7). These results define a consensus polypeptide sequence which plays an essential role in the rate-limiting activation of p53. Specifically, substitution of any of the positively charged amino acids (K370, K372, K373, R379, K381, or K382) with alanine dramatically reduces the ability of the 19 amino acid synthetic peptide to potently activate p53. Substitutions of the remaining nine amino acids with alanine are tolerated and do not dramatically reduce the apparent K_m of activation. These results define the exact molecular requirements and assay conditions to develop small molecular weight regulators of the p53 allosteric activation pathway.

DnaK targets the C-terminal regulatory site of human p53.

DnaK can activate latent, wild-type and some mutant forms of p53 protein for sequence-specific DNA binding (Hupp et al, 1993). Although the mechanism is undefined, it is clear that the tetramerization status of p53 is preserved after activation, indicating that DnaK does not dissociate tetramers into smaller units. DnaK activation of p53 also requires energy in the form of heat, as the reaction cannot proceed at 0°C (see below). In addition, DnaK partner proteins DnaK and GrpE are not required for this interaction, reducing the likelihood that substrate targeting and turnover, putatively associated with GrpE and DnaK function, are required for DnaK function in this system. Given the simplicity of the two component activation reaction driven by DnaK protein, studying the mechanism of p53 interaction with DnaK may not only provide insight into how p53 protein activity is altered by interacting proteins, but it may give insight into the

mechanism whereby DnaK interacts with a native protein substrate.

Modification of a C-terminal negative regulatory domain on latent p53 has been shown to trigger the activation of DNA binding by other proteins: Binding of the monoclonal antibody PAb421 or phosphorylation by either casein kinase II, protein kinase C, or cdc/cyclins can activate latent p53, whereas the monoclonal antibody ICA-9 binding to the casein kinase II site can reverse the activation (Figure 15). As the target site for activation of p53 by DnaK has not been determined, we investigated a possible interaction of DnaK with the C-terminal regulatory domain.

A series of synthetic peptides was screened for the ability to interfere with the activation of human p53 DNA binding by DnaK. Reactions were divided into two stages by first incubating p53 and DnaK in the absence or presence of synthetic peptides at 30°C and then assaying for sequence-specific DNA binding at 0°C. Synthetic peptide 379-393 derived from the C-terminus of p53 competed effectively with DnaK (Fig. 9A, lanes 8-10 versus lane 3). The concentration of DnaK in the reaction was 1.4 μM and the amount of peptide required for 50% inhibition of DnaK activity in this assay was approximately 0.5 to 1 μM. These results suggest that DnaK protein targets the C-terminal domain of p53. In addition, the requirement for stoichiometric amounts of inhibitory peptide suggest that, although DnaK protein is required in a 50-fold molar excess over p53 protein, inhibition of most of the DnaK is required to block p53 activation. The requirement for high levels of DnaK protein may depend on an altered conformation of DnaK at higher protein concentrations, as in one biochemical system, it is the absolute DnaK protein concentration and not the molar ratio of DnaK to its substrate that dictates chaperone activity. Indeed, the specific activity of HSP70 isoforms may be sensitive to

changes in their conformation or oligomeric structure (Blond-Elguindi, 1993).

The inhibition by peptide 379-393 is specific for DnaK since PAb421 (lanes 5-7 versus lane 2) and casein kinase II (lanes 11-13 versus lane 4) activation of p53 were not blocked by this peptide. Peptide 379-393 was unable to compete with casein kinase II activation of p53 despite harboring the phosphorylation site for this kinase. Short synthetic peptides containing the casein kinase II phosphorylation site of p53 have only a relatively weak homology to the defined consensus motif of the casein kinase II site and have previously been shown to be ineffective as substrates for this enzyme. Additional structural determinants within the p53 homotetramer may contribute to casein kinase II specificity.

The inability of a flanking peptide harboring the PAb421 antibody epitope ((Stephen et al, 1995); peptide 369-383) to compete with DnaK activation indicates that peptides at stoichiometric concentrations do not in general inhibit the biochemical function of DnaK in this reaction (Fig. 9B, lanes 4-6). Peptide 369-383 is, however, biochemically active as it did inhibit p53 activation by PAb421 (Fig. 9B, lanes 1-3). Peptide 374-388 was also effective in competing with DnaK activation of p53 (Fig. 9C, lanes 6-9 versus lane 1). together, these data indicate that amino acids 389-393 and 374-378 do not contribute to DnaK specificity while amino acids 379-388 play an important role in p53 recognition by DnaK (figure 15). Thus, this suggests that one important step in DnaK activation of p53 involves an interaction with a motif situated in between the casein kinase II and protein kinase C phosphorylation sites.

DnaK binds directly to synthetic peptides derived from the p53 C-terminus.

We examined whether the synthetic peptides derived from the C-terminus of p53 were inhibiting activation of p53 protein by DnaK due to direct binding to DnaK. A slightly different subset of peptides was chosen for this analysis in order to define a minimal site of interaction. DnaK and the respective peptides were co-incubated at 30°C, bound peptide was separated in a native polyacrylamide gel assay, and analyzed by Western blot via streptavidin binding to the biotinylated peptides (figure 10). DnaK did not interact with peptide 366-380, but showed weak binding to peptide 371-385. Thus, amino acids 381-385 define an important component of the DnaK binding site. Peptide 376-390 formed a stoichiometric complex with DnaK and exhibited the strongest binding to DnaK. The peptide 379-393 bound weaker to DnaK, as the presence of the last three acidic amino acids in the peptide may reduce the stability of complex formation with DnaK. Therefore, the last few amino acids do not contribute to the DnaK binding site, but rather seem to reduce binding. This is consistent with the fact that DnaK is a more potent activator of latent p53 protein when the final four amino acids are removed (Hupp and Lane, 1995) and that peptide 379-393 has a slightly reduced inhibitory activity towards DnaK in comparison with peptide 374-388 (Fig. 9C lane 4 versus lane 8). Together, these results indicate that DnaK can form a stable and stoichiometric complex with specific p53 peptides and refine assignment of the DnaK interaction site on p53 protein to amino acids 381-388 (figure 15).

Interaction of human Hsc70 protein with p53 C-terminal peptides.

Bacterial DnaK belongs to the heat shock protein 70 family and is very homologous to its human members (Table 2).

Although HSP70 homologues can exhibit quite distinct substrate specificities, peptides that bind to bacterial DnaK with a high affinity contain some similar hydrophobic properties that match the consensus binding sequence for some mammalian heat shock proteins, suggesting the possibility that some conservation of substrate specificity may exist. As such, we investigated whether mammalian HSP70 isoforms can also bind directly to peptides derived from the C-terminal regulatory domain of human p53. HSP70 family members studied include Grp79, Hsc70, and Hsp70 (see below).

The constitutively expressed Grp78/BiP is a resident protein of the endoplasmic reticulum (ER) and a member of the glucose-regulated protein family. An affinity panning approach of peptide libraries in bacteriophages has identified an optimal heptameric motif for the molecular chaperone BiP with a high content of aromatic and hydrophobic amino acids. Recombinant hamster Grp78 (BiP), however, did not show any binding to our C-terminal p53 peptides (Table 2). The 70kDa heat shock cognate protein, Hsc70, is a constitutive member of the Hsp70 family found in the nucleus and cytosol, just like stress-induced Hsp70 (Gething et al, 1992). Human Hsp70 and Hsc70 proteins have been well characterized biochemically with respect to their ability to interact with denatured protein substrates (Freeman et al, 1995). Although human Hsp70 protein did not bind to the C-terminal p53 peptides (Table 2), human Hsc70 protein exhibited strong binding to the same peptides as observed with DnaK (figure 11). However, Hsc70 protein binding to the C-terminal p53 peptides was less avid than that observed for DnaK, which has been inferred previously, based on the relative ability of a C-terminal p53 peptide to compete with bovine Hsc70 and DnaK protein binding to unfolded lactalbumin.

The functional significance of this conserved interaction of DnaK and Hsc70 with the C-terminal p53 peptide is not yet clear. Evidence that mammalian Hsp/c70 proteins may modulate the p53 pathway comes from the observation that overexpression of Hsc70 protein can suppress focus formation of rat fibroblasts induced by mutant p53 plus *ras* or *myc* plus *ras* and mammalian Hsc70/Hsp70 binds to mutant p53 synthesized in reticulocyte lysates unless the C-terminal 28 amino acids have been removed. Our data indicate that one interaction site for human Hsc70 protein may reside in the p53 protein C-terminus and that human Hsp70 does not interact similarly. Despite the similarity of Hsc70 and DnaK in binding to C-terminal human p53 peptides, Hsc70 was not able to substitute for DnaK in activating latent human or murine p53 for DNA binding (data not shown). This suggests that: (1) another factor may be required to function in concert with Hsc70 to activate p53 protein; (2) that the reduced affinity of Hsc70 is sufficient to preclude activation of p53 protein; (3) that DnaK protein has at least one other recognition site on p53, and (4) DnaK activation is not directly related to binding to the C-terminal region.

DnaK binds with a lower affinity to C-terminal peptides derived from murine p53.

Murine p53 is 85.8% homologous to human p53 and both proteins include well described functional features like the DNA binding domain in the central region and a C-terminal tetramerization domain (Wang et al, 1994). Both proteins can activate transcription of target genes containing p53 binding sites (Farmer et al, 1992) and they can both function as transcription factors in yeast (Scharer et el, 1992). Human and murine p53 also form hetero-tetramers when co-translated. The cellular human proteins TBP (TATA-binding protein) and hdm2 (human double minute 2) also bind murine p53. However, although murine

and human p53 have conserved their C-terminal cdc2/cyclin, protein kinase C and casein kinase II phosphorylation sites, there is a striking degree of divergence in the region of human p53 implicated in binding to DnaK protein (figure 15).

Peptides from the murine p53 C-terminus bind much weaker to DnaK protein than the peptide from the human p53 sequence (figure 12), suggesting that this region does harbour a weaker DnaK interaction site. There are four amino acid changes in murine p53 compared to the human DnaK binding site (figure 15) and based on the HSP70 isoform peptide binding specificity defined by Fourie et al, (1994) the L to T, P to V, and T to K amino acid substitutions in the C-terminus of murine p53 may be sufficient to reduce DnaK binding. However, a similarity could be detected in terms of the relative affinity of binding pattern of murine C-terminal peptides: Peptide 368-382 which localizes between the protein kinase C and casein kinase II phosphorylation sites showed a more pronounced binding than the neighbouring peptides. If the C-terminus is a major interaction site of p53 protein for DnaK, then the reduced ability of DnaK to bind to murine p53 peptides should also be reflected in a reduced specific activity of DnaK in its activation of latent murine p53 protein. Addition of increasing concentrations of DnaK resulted in only a very weak activation of the DNA binding activity of latent murine p53 (figure 13), under conditions where PAb421 gave potent activation. These results are consistent with the hypothesis that the C-terminus of latent human p53 protein harbours a DnaK recognition motif.

Mechanism of DnaK activation of murine p53.

To confirm whether the C-terminus of p53 harbors a DnaK recognition motif, the ability of DnaK to activate latent murine p53 derived from insect cells was also tested.

Although murine latent p53 from *E. coli* was not activated by DnaK, latent murine p53 from insect cells was very efficiently activated *in vitro* by DnaK (figure 14A). As insect cells contain the three conserved kinases known to activate p53 (including cdc2/cyclin, protein kinase C, and casein kinase II) and as rat p53 produced in insect cells is heavily phosphorylated at the cdc2 and casein kinase II phosphorylation sites, it is likely that *in vivo* phosphorylation of murine p53 protein synthesized in insect cells may account for the increased activation seen with DnaK. The results above show that such a "priming" of latent, human p53 protein by a kinase renders the latent, phosporylated tetramer more receptive to activation by weak activators.

If this "priming" of murine p53 protein by *in vivo* phosphorylation at the cdc2 or casein kinase II site was responsible for the ability of DnaK to activate latent, murine p53, then dephosphorylation of the protein would presumably reduce the extent of activation by Dnak protein. One way to address this is to dephosphorylate *in vitro* the purified p53 protein. However, protein phosphatase dephosphorylate *in vitro* the purified p53 protein. However, protein phosphatase dephosphorylation at the cdc2 and casein kinase II sites is very inefficient *in vitro*, precluding such an analysis. Instead, we chose to produce a cdc2 serine to alanine point mutation in the murine p53 protein which would preclude phosphorylation at this site *in vivo*. The murine p53Ala309 mutant protein was significantly (50-65%) less active than wild-type p53 after incubation with DnaK protein (figure 14). This indicates a concerted synergistic action between binding of a factor at the C-terminal regulatory domain and phosphorylation at the cdc2 site. In fact, PAb421 was still efficient in activating insect cell-derived murine p53 protein mutated at this site (Ala309).

Identification of a second regulatory domain on p53 whose modification contributes to activation.

These data suggest that a second regulatory domain exists in the C-terminus of p53, whose modification can lead to p53 activation. Since the binding of monoclonal antibodies often mimics the action of cellular factors and can regulate protein function *in vitro*, we investigated the activity of latent murine p53 after incubation with the monoclonal antibody PAb241, whose epitope resides within amino acids 288-297 and is close to the cdc2 phosphorylation site. Antibody PAb241 is indeed able to activate latent, murine p53 for DNA binding (figure 14B), although the efficiency of activation is 40% of the activation by PAb421.

Discussion

A rate-limiting step in the activation of p53 sequence-specific transcriptional function in vivo.

Prior to these studies, key chemical steps involved in activating directly p53 protein following DNA damage in mammalian cells have been undefined. However, the ability to activate the sequence-specific transcriptional function of wild type p53 in vivo, using a monoclonal antibody (Pab421) known to activate the specific DNA binding function of p53 in vitro, underscores the rate-limiting role of this allosteric activation reaction in modulating the p53 pathway. These data establish the presence of a pool of latent p53 which can be activated post-translationally in normal cells and demonstrate that the activation of p53-responsive reporter genes can occur in the absence of other signals that might be mediated by DNA damage within the cells; ie direct affects on the template DNA. Indeed, a p53-dependent superinduction of $p21^{CIP1}$ mRNA was observed upon serum stimulation of quiescent wild type

fibroblasts in the presence of cycloheximide (Macleod et al 1995), suggesting that activation of p53 transcriptional function can occur in the absence of protein synthesis. In addition, the fact that modification of p53 in the C-terminal negative regulatory domain resulting in loss of the antibody Pab421 epitope (Milner 1984, Ullrich et al 1992; Hupp and Lane 1995) or alternative splicing of mouse p53 mRNA resulting in removal of the negative regulatory domain occurs in tissue culture systems (Arai et al 1986, Kulesz-Martin et al 1994), indicates that modification of the negative regulatory domain can occur in vivo in the absence of DNA damage.

That activation of the transcriptional activity of p53 can occur without an increase in p53 protein levels is consistent with the existence of distinct cellular signalling pathways capable of modulating the conversion between latent and activated forms of p53 (Hupp and Lane 1995). The results also explain how microinjection of antibodies to the carboxy-terminus of p53 can block cell cycle progression (Mercer et al 1982; Mercer et al 1984; Deppert et al 1990), by virtue of activating the biochemical function of endogenous p53 protein. As such, determining the mechanism of p53 activation may have future therapeutic relevance. Selective activation of wild type p53 by agents that do not induce DNA damage may differentially alter the response of normal tissue as compared to p53-negative tumour cells to therapeutic agents toxic only to rapidly cycling tumour cells. On the other hand one class of mutation in p53 results in proteins that cannot be activated after phosphorylation, but can be activated in vitro by binding of the activating monoclonal antibody Pab421 (Hupp et al 1993; Niewolik et al 1995). The latter study is notable, as almost one-third of mutant forms of p53 synthesized in human tumour cell lines can be activated in vitro by the binding of Pab421, suggesting that a considerable proportion of human tumours might

respond to allosteric activators by rescuing their p53 response.

An allosteric model for the activation of p53.

The mechanism of p53 latency and the mechanism whereby the protein is activated after covalent modification is not known. In addition, neither the crystal structure of the full length unphosphorylated p53 tetramer nor the phosphorylated tetramer are known, thus precluding predictions on the mechanisms of latency and activation. However, using synthetic peptides derived from the C-terminal negative regulatory, domain of p53 we have defined a small molecule which can activate latent p53 function and this information has led to a specific biochemical model reflecting a possible mechanism of p53 latency and mechanism whereby phosphorylation alters polypeptide structure. The key elements of this model are that the C terminal tails of p53 interact with the core of the molecule (Figure 8). This interaction locks the core into a conformation that is inactive for DNA binding. When this tail-core interaction is disrupted by covalent modification, non-covalent modification or deletion, the core is now able to adopt the active form. Complete activation of p53 is not accomplished effectively by synthetic polypeptides alone, suggesting that negative regulatory domain interactions are not easily disrupted. The stability of the negative regulatory domain interactions are also manifested in the ability to purify to homogeneity latent p53 tetramers using three sequential chromatographic matrices (Hupp and Lane 1994a); ie a spontaneous shift in equilibrium from the latent to activated state during purification would have reduced latent tetramer yield if p53 negative regulatory domain-tetramer interactions were of low affinity and subject to destabilisation.

Although the activating peptide was not able to potently activate latent p53, a striking decrease in the apparent K_m for peptide activation and an increase in the specific activity of p53 was accomplished by a prior modification of the C-terminus by casein kinase II or interactions with the heat shock protein DnaK under conditions in which activation is rate-limiting. This modification presumably "primes" p53 protein and decreases the energy barrier to peptide-activation by shifting the equilibrium to a state more favourable to stabilize synthetic peptide-tetramer interactions (Figure 8). This model is also supported by the existence of mutant forms of p53 which can be activated from their latent state by the Pab421 monoclonal antibody, but cannot be activated after phosphorylation of its negative regulatory domain (Hupp et al 1993). Thus, a single point mutation in the core domain of p53 can stabilize C-terminal negative regulatory domain interactions and hinder activation by covalent modification.

Peptide synergism with activating factors also questions the reliability of using immuno-affinity purified p53 to interpret biochemical studies on its post-translational regulation. Immunoaffinity purification of p53 using a Pab421 antibody-linked matrix utilizes a peptide similar to the activating peptide C_1 (Figure 3) to elute p53 from the antibody column (Bargonetti et al 1991). That specific peptides can synergise with p53-activating factors (Figures 4-6), indicates that conclusions based on studies of regulation of p53 by enzymes may be misleading due to contamination of protein preparations with the peptides.

The allosteric model of negative regulation model can also be used to predict that reduction in net basic charge in the vicinity of the carboxy-terminal regulatory site would be destabilising and could displace the negative regulatory domain from its binding site. Apart from phosphate

modification, three other independent experiments support this prediction. First, site directed mutagenesis producing the substitution of four basic amino acid residues with hydrophobic amino acids from the C-terminal negative regulatory domain has already been shown to strikingly increase the specific activity of p53 as a sequence-specific transcriptional activator *in vivo* (Tarunina and Jenkins 1993), presumably by allowing spontaneous activation of p53 during tetramer assembly in cells. These latter results are striking, especially as this quadruple regulatory site mutant form of p53 actually has a higher specific activity than p53 activated by deletion of its negative regulatory domain. Second, a monoclonal antibody, which binds to the acidic domain in the extreme C-terminus, cannot activate p53 and appears to actually stabilize negative regulatory domain interactions (Hupp and Lane 1994a). Third, using an elegant approach, random mutagenesis of the C-terminus of human p53 and subsequent screening for mutants which have lost sequence-specific transcriptional activity in yeast model systems resulted in acquisition of two independent point mutations in the casein kinase II site at amino acid 393 which increased net basic charge (Ishioka et al 1995); an expected result if maintenance of net basic charge were an important element in contributing to p53 latency.

In conclusion, peptide-dependent activation provides the opportunity to formulate biochemical models of p53 latency which can be tested experimentally and thus shed new light on the role phosphorylation plays in allosterically altering polypeptide conformation. These studies also hold promise for the design of low molecular weight agents which can also activate mutant forms of p53 defective in allosteric activation by protein kinases, as activation of wild type p53 can now be shown in vivo using a monoclonal antibody.

Definition of an Hsc70 binding site on p53.

The role of heat shock proteins in the p53 regulatory pathway is not fully understood and various reports have suggested that an interaction between the HSP70 protein family and p53 may be specific and of significance. Initial studies suggested a link with mutated p53, as in tumour cell lines, Hsp/c70 proteins immunoprecipitates with a fraction of mutant, but not wild-type p53 protein. In addition, overexpression of Hsc70 can suppress focus formation of cell lines induced by mutant p53 protein. The binding of rabbit Hsp/c70, from reticulocyte lysates to p53 protein requires the last 28 amino acids of murine mutant, not wild-type p53, but these data did not establish whether this C-terminal region can directly bind to HSP70 isoforms or if p53 conformation is altered by the deletion thus precluding HSP70 binding.

More recent studies have shown that the p53 protein, induced by heat treatment of cells with a wild-type p53 gene, localizes in the cytoplasm, where it forms a complex with Hsc70. The data presented in our study localizes one putative binding site of Hsc70 to the extreme C-terminus of native, wild-type p53 and is based on direct binding of peptides derived from the C-terminus of p53.

A physiological role for the interaction of p53 protein with heat shock proteins and whether a transient interaction with an HSP70 family member *in vivo* is sufficient to activate p53 protein remains to be established. This latter hypothesis find support from the observation that mutating all known regulatory phosphorylation sites on human p53 does not alter its biological function in human cells, suggesting that some other factor may be involved in p53 activation in cells. A second interesting question to address is whether the Hsc70 interaction with mutant p53 in tumour cells is a

result of the altered conformation of the mutant protein or whether the mutant conformation is a consequence of the interaction with and unfolding by Hsc70 and its associated partner proteins. With one putative binding site of human Hsc70 localized on human p53 and the observation that human Hsp70 may not be involved in this process, this site can now be specifically targeted *in vivo* to approach these questions.

Definition of a DnaK binding site on p53.

The bacterial heat shock protein DnaK is a potent activator of latent human p53 and allosteric-class of mutant p53 proteins *in vitro*. Since other activators (like PAb421, protein kinase C, and casein kinase II) also target the extreme C-terminal regulatory domain of p53 and another activator (cdc2/cyclin kinases) targets a site flanking the tetramerization domain, we tried to determine if the site of interaction with DnaK was also in this region. We have identified synthetic peptides derived from the extreme C-terminal negative regulatory domain of p53 which compete effectively with DnaK activation of p53, suggesting that the C-terminal regulatory site of p53 contains an important interaction site for DnaK. We were unable to show effective inhibition of DnaK activation of latent p53 by synthetic peptides derived from the amino-terminal region of p53 at μM levels observed using carboxy-terminal peptides (data not shown), suggesting that the C-terminal regulatory site may contain the major interaction site for DnaK. A combination of the peptide inhibition studies (Figure 9) and the direct peptide binding studies (Figure 10) has led to the refinement of the DnaK binding site as amino acids 381-388 on p53. However, we have not yet shown a stable complex between DnaK and native, wild-type p53 protein, but if the interaction is transient or relatively weak, stable complexes may not exist.

The specificity of DnaK-peptide binding has been studied before, using a peptide display library. Short peptides with internal hydrophobic and N-terminal positively charged residues were shown to be beneficial for binding by DnaK, whereas negatively charged amino acids only bound poorly. The peptide KKLMFKT, which corresponds to the DnaK binding side on p53, has been shown to compete efficiently with the binding of reduced and carboxymethylated lactalbumin (RCMLA) to DnaK. Indeed, by changing the second amino acid to a tyrosine (KYLMFKT) the binding of bovine Hsc70, which only bound moderately to the original peptide, changes dramatically to reach the same degree as binding to DnaK. In murine p53 the sequence is changed in that particular region to KKTMVKKV, which reduces the affinity of DnaK to the target sequence. Thus, the exchange of three hydrophobic residues and the introduction of a positive C-terminal charge reduces the affinity, but do not abolish critical amino acids for DnaK binding.

However, unphosphorylated murine p53 produced in bacterial is not efficiently activated by DnaK, unlike human p53 (figure 13), suggesting that mutation of these amino acids may effect the affinity of DnaK interaction with the native p53 tetramer.

Mechanism of activation of murine p53 by DnaK.

Whereas human and murine p53 are very similar on a structural level, they may respond differently to post-translational modifications that target the C-terminus. The carboxy-terminal serine 392 site of human p53, which is phosphorylated by casein kinase II *in vivo* and *in vitro*, does not contribute to wild-type human p53's biochemical and biological growth suppressor activity. On the other hand, a mutation of the casein kinase II site of murine p53 inactivates its ability to suppress growth in rodent cell lines. Multiple evolutionary changes in amino acids

between human and murine p53 in the extreme C-terminus suggest that this region of p53 has evolved slightly distinct controlling mechanisms despite having conserved the cdc2/cyclin, protein kinase C, and casein kinase II phosphorylation sites.

The activation of murine p53 from insect cells by DnaK represents a new model system, which helps to describe the mechanism of activation for DNA binding of murine p53. the activation of murine p53 by DnaK appears to be similar to the model proposed previously for activation of human p53 by phosphorylation, in which phosphorylation of latent human p53 can "prime" the protein and convert it to a form that can be activated by weak activators, like peptides. Similarly, DnaK is a weak activator of unphosphorylated, murine p53, due to differences in amino acids of the binding region reflected in its reduced affinity for murine peptides. Phosphorylation of murine p53 in insect cells, may prime for a more efficient activation by DnaK. Wang and Prives (1995) have shown that phosphorylation by cyclin B/cdc2 and cyclin A/cdk2 complexes can lead to increased DNA binding by human and murine p53. Mutation of serine 309 to Alanine precludes cdc2 phosphorylation of murine p53 *in vivo* and reduces the activation by DnaK by approximately 65%. Comparably the potential of PAb421, a strong activator in this system, to activate for DNA binding of Ala309 is only reduced by 15-30%. Thus, there is cooperation between these two sites for activation affecting both, weak (DnaK) and strong activators (PAb421), but weak activators are more dependent of the effect of cooperation. Furthermore, the monoclonal antibody PAb241 imitates the effect of cdc2 phosphorylation and can also activate murine p53 for DNA binding. Thus, our results reveal a second regulatory domain within p53 in close proximity to the cdc2 site of phosphorylation. Since tumour-derived, inactive mutant p53 protein can be activated for sequence-specific transcription in tumour

cells by modification of the negative regulatory domain I (Figure 15), identification of a second regulatory site whose modification activates p53 may lead to another related approach to reactivate mutant p53 proteins.

5

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Table 1

Experiment 1

Injected Material	β-galactosidase positive cells	Number of cells injected	% β-galactosidase positive cells
Buffer	15	105	14
Buffer	3	117	3
PAb 421	54	119	45
PAb 421	75	181	41
PAb 246	12	215	6
PAb 246	6	70	9

Experiment 2

Injected Material	β-galactosidase positive cells	Number of cells injected	% β-galactosidase positive cells
Buffer	17	216	8
Buffer	14	184	7
PAb 421	90	223	40
PAb 421	47	166	28
PAb 246	0	140	0
PAb 246	3	204	2

Table 2

Heat shock protein	Similarity to DnaK in %	Identity to DnaK in %	Binding to p53 C-terminal peptides 366-380	371-385	376-390	379-393
DnaK	100	100	-	++	+++	++
human Hsc70	66.8	49.0	-	++	+++	+++
human Hsp70	66.9	50.1	-	+	+/-	+/-
human Grp78/ BiP	69.2	51.2	- (hamster)	-	-	-
human Grp75	75.0	60.4	n.d.	n.d.	n.d.	n.d.

CLAIMS:

1. A substance which has the property of activating the sequence specific DNA binding activity of latent p53, said substance consisting of:

(i) a fragment of the C-terminal regulatory domain of p53 protein, or an active portion or derivative thereof;

(ii) a fragment of murine p53 including the epitope bound by antibody Pab241, or a fragment from the corresponding region of human p53, or an active portion or derivative thereof; or,

(iii) a functional mimetic of (i) or (ii) above;

wherein the fragment or mimetic is optionally coupled to a functional moiety.

2. The substance of claims 1 wherein fragment of the C-terminal regulatory domain of p53 protein consists of amino acids 369 to 383.

3. The substance of claim 1 or claim 2 wherein the fragment of the C-terminal regulatory domain of p53 includes residues corresponding to the amino acids K370, K372, K373, R379, K381, K382.

4. The substance of claim 1 wherein the epitope of antibody Pab241 is localised between amino acid residues residues 287 to 310 of murine p53, corresponding to residues 293 to 316 of human p53.

5. The substance of any one of claims 1 to 4 wherein the functional moiety is a label, a drug, or a carrier molecule.

6. The substance of claim 5 wherein the functional moiety is a 16 amino acid peptide sequence derived from the homeodomain of *Antennapedia*.

7. The substance of claim 5 wherein the functional moiety is a molecule which enhances the p53 activating effect of the substance.

8. A pharmaceutical composition comprising one or more of the substances of any one of the the preceding claims.

9. The pharmaceutical composition of claim 8 in combination one or more p53 activators.

10. The composition of claim 9 wherein the p53 activator is a heat shock protein such as DnaK, antibody Pab421, antibody Pab241 and/or casein kinase II.

11. The substance of any one of claims 1 to 7 for use in a method of medical treatment.

12. The use of the substance of any one of claims 1 to 7 in the preparation of a medicament for activating latent p53.

13. The use of claim 12 wherein the medicament is for the treatment of cancer or other hyperproliferative disorders.

14. A substance which has the property of binding to DnaK, said substance consisting of a fragment of the C-terminal regulatory domain of p53 protein from amino acid residues 381 to 388 (KKLMFKT), or an active portion or derivative thereof.

15. Nucleic acid encoding a substance of any one of claims 1 to 7 or 13.

16. A vector comprising the nucleic acid of claim 15 operably linked to control sequences to direct its expression.

17. The use of a substance of any one of claims 1 to 7 or 13 in screening for (i) compounds having one or more of the biological activities of the substances described above or (ii) compounds which are binding partners of one of the substances.

18. The use of a fragment of p53 including (i) amino acid residues corresponding to K370, K372, K373, R379, K381, and K383 or (ii) amino acid residues comprising the epitope of murine p53 bound by antibody Pab241, or a fragment from the corresponding region of human p53, in the design of an organic compound which is modelled to resemble the three dimensional structure of said amino acid residues, the organic compound having the property of activating latent p53.

19. The use of a fragment of p53 including amino acid residues corresponding to 381-388 (KKLMFKT) of p53, in the design of an organic compound which is modelled to resemble the three dimensional structure of said amino acid residues, the organic compound having the property of binding to DnaK.

Fig.1A.

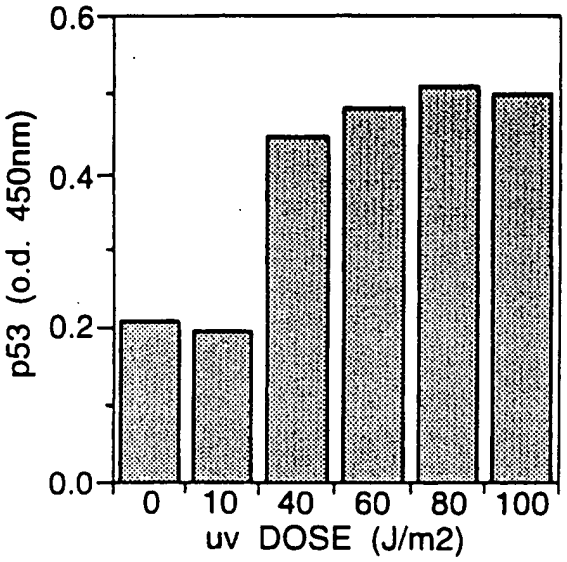


Fig.1B.

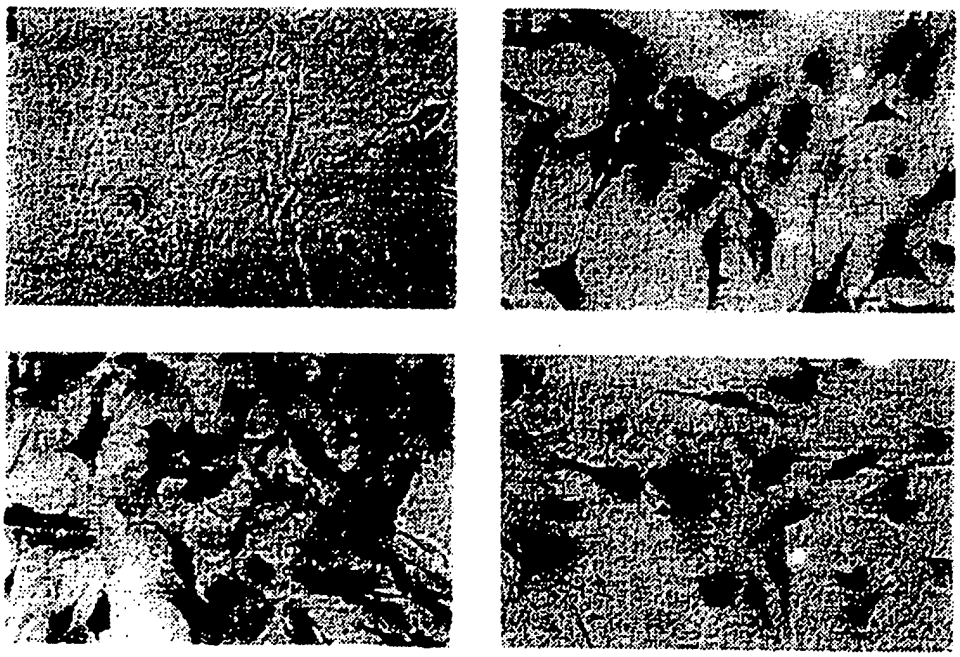


Fig.2.

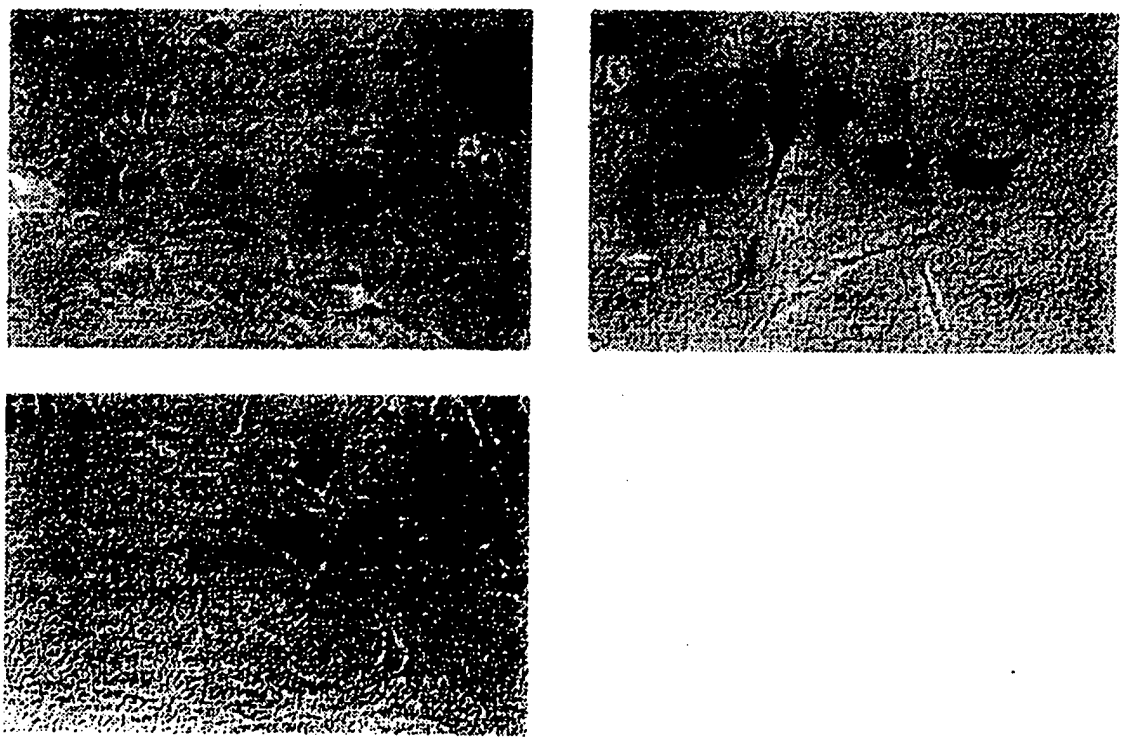
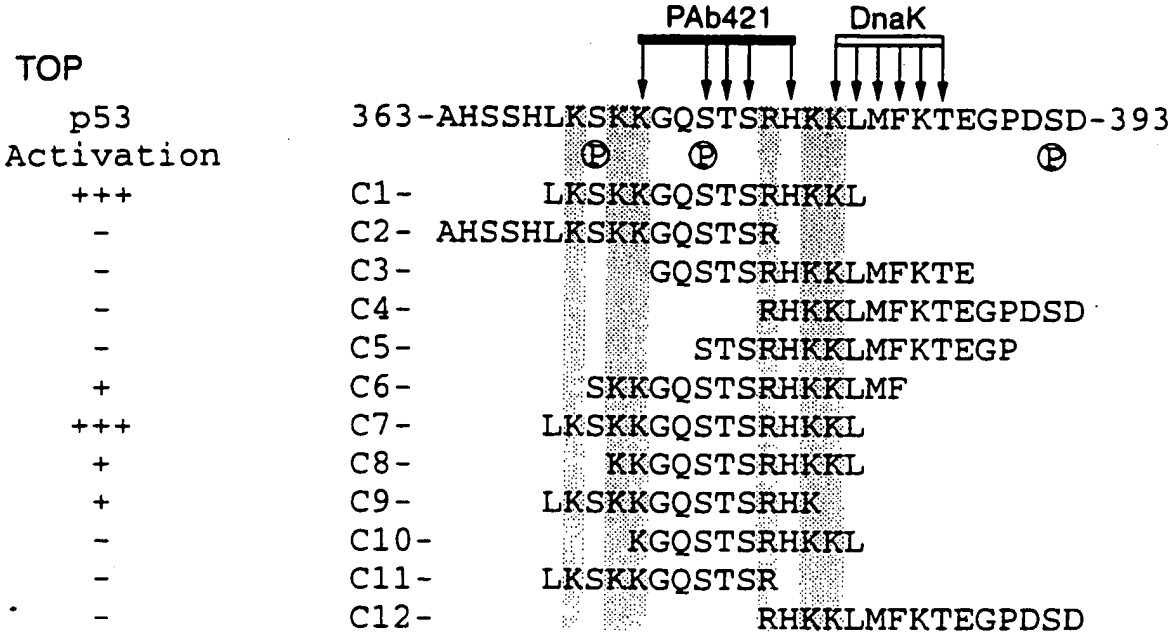


Fig.3.


PAb421
DnaK
TOP
p53
Activation
363-AHSSHLKSKKGQSTSRHKKLMFKTEGPDSD-393
+++ C1- LKSKKGQSTSRHKKL
- C2- AHSSHLKSKKGQSTSR
- C3- GQSTSRHKKLMFKTE
- C4- RHKKLMFKTEGPDSD
- C5- STSRHKKLMFKTEGP
+ C6- SKKGQSTSRHKKLMF
+++ C7- LKSKKGQSTSRHKKL
+ C8- KKGQSTSRHKKL
+ C9- LKSKKGQSTSRHK
- C10- KGQSTSRHKKL
- C11- LKSKKGQSTSR
- C12- RHKKLMFKTEGPDSD

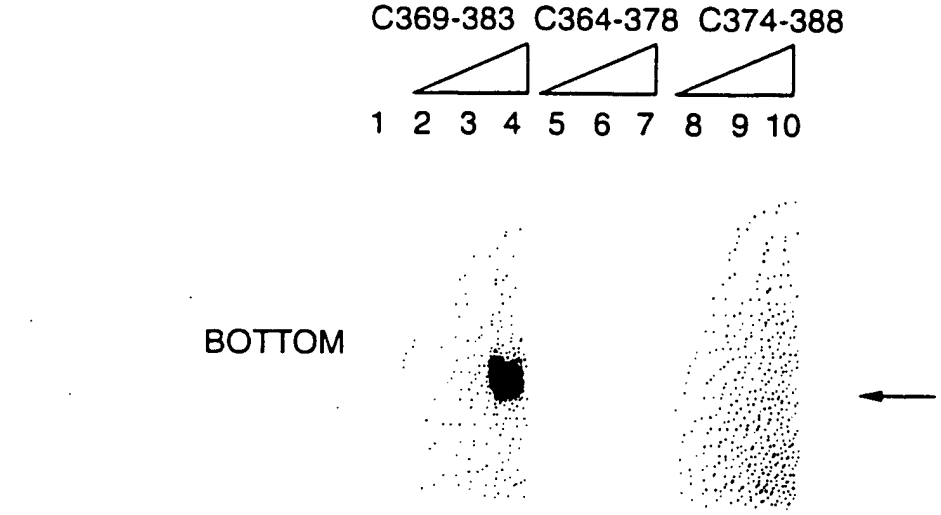

C369-383
C364-378
C374-388
1 2 3 4 5 6 7 8 9 10
BOTTOM

Fig.4.

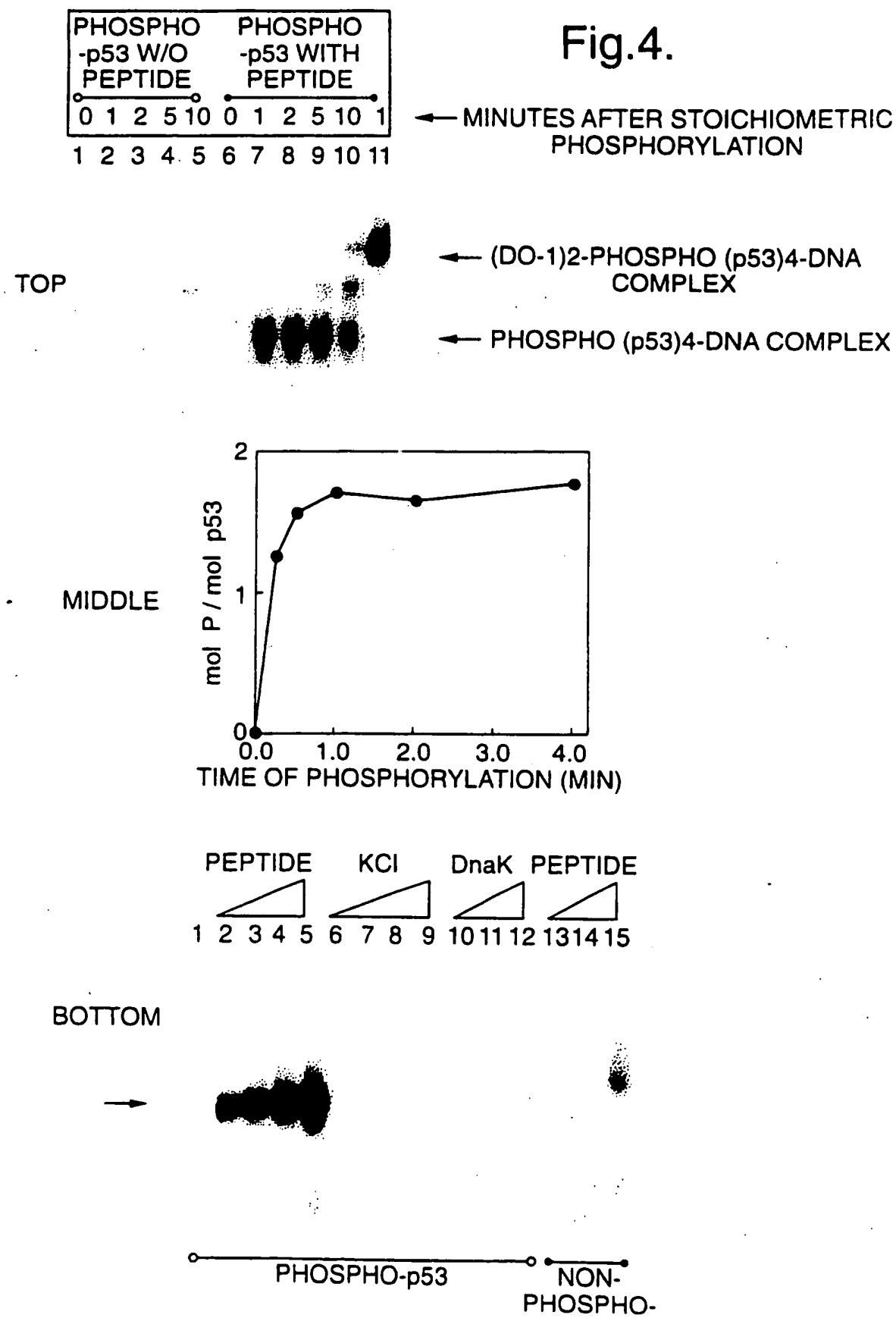

PHOSPHO -p53 W/O PEPTIDE
PHOSPHO -p53 WITH PEPTIDE
0 1 2 5 10 0 1 2 5 10 1
1 2 3 4 5 6 7 8 9 10 11
MINUTES AFTER STOICHIOMETRIC PHOSPHORYLATION
TOP
(DO-1)2-PHOSPHO (p53)4-DNA COMPLEX
PHOSPHO (p53)4-DNA COMPLEX
MIDDLE
mol P / mol p53
0
1
2
0.0 1.0 2.0 3.0 4.0
TIME OF PHOSPHORYLATION (MIN)
PEPTIDE KCl DnaK PEPTIDE
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
BOTTOM
PHOSPHO-p53
NON-PHOSPHO-p53

Fig.5.

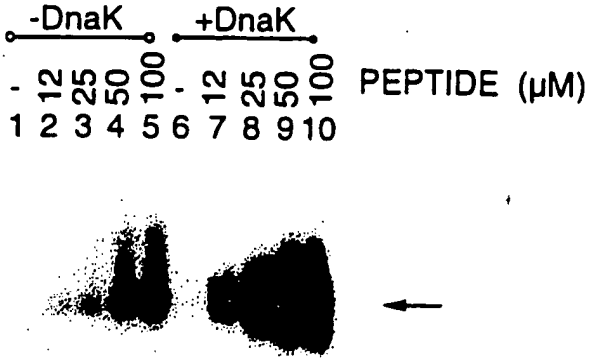
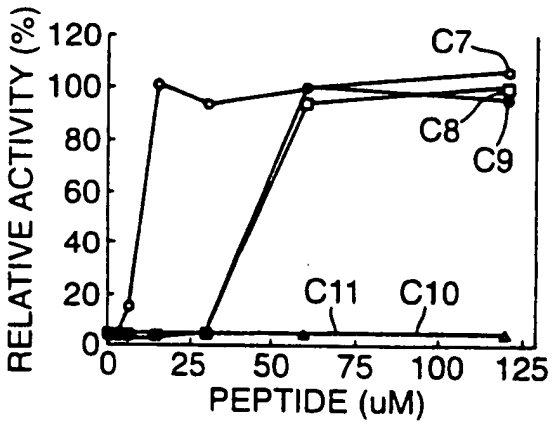


Fig.6.



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

Fig.7.

ALANINE SUBSTITUTED PEPTIDES

1-AKSKKGQSTSRHKKL
2-LASKKGQSTSRHKKL
3-LKAKKGQSTSRHKKL
4-LKSAKGQSTSRHKKL
5-LKSKAGQSTSRHKKL
6-LKSKKAQSTSRHKKL
7-LKSKKGASTSRHKKL
8-LKSKKGQATSRHKKL
9-LKSKKGQSASRHKKL
10-LKSKKGQSTARHKKL
11-LKSKKGQSTSAHKKL
12-LKSKKGQSTSRAKKL
13-LKSKKGQSTSRHAKL
14-LKSKKGQSTSRHKAL
15-LKSKKGQSTSRHKKA
16-LKSKKGQSTSRHKKL

Fig.8.

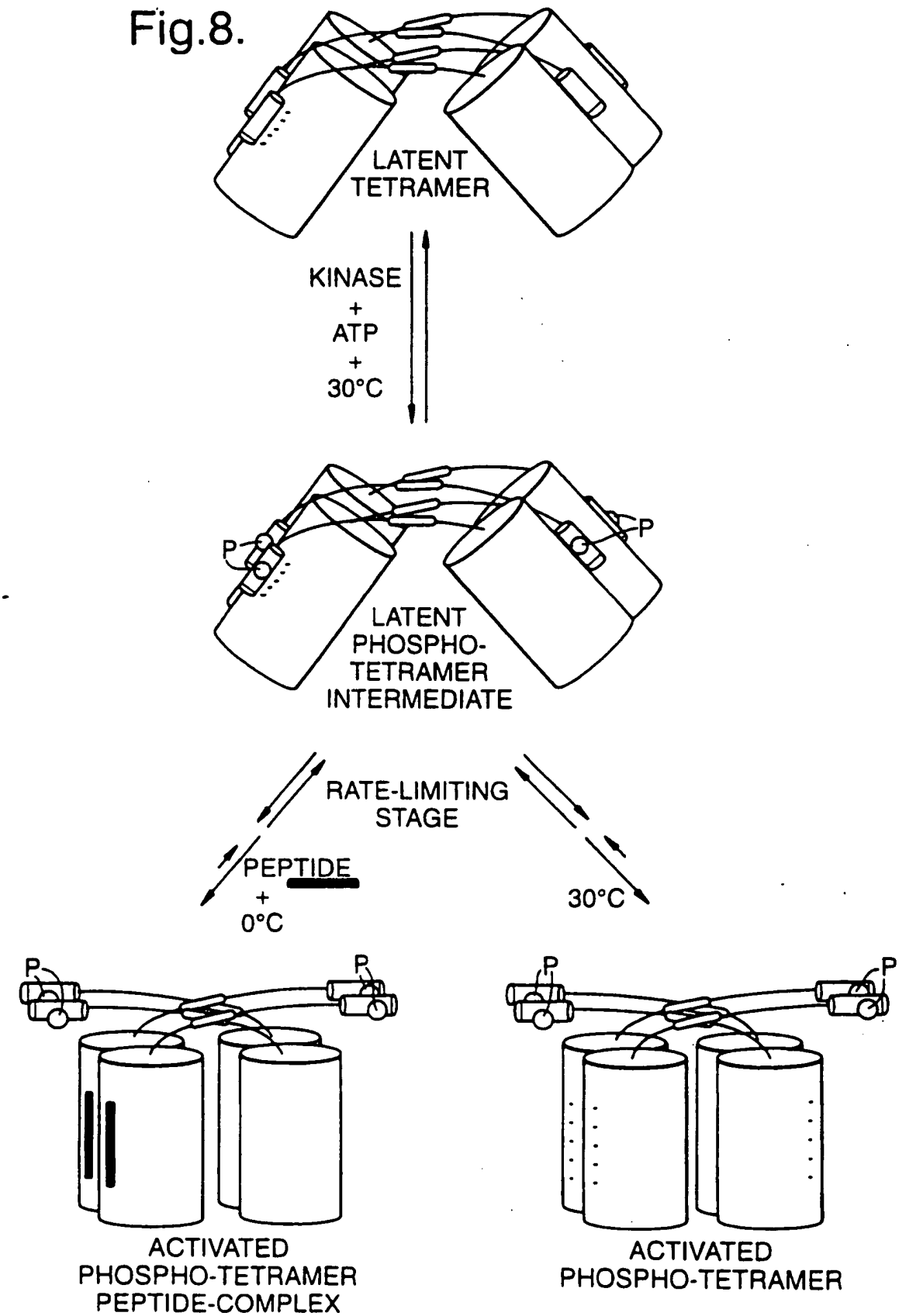

LATENT
TETRAMER
KINASE
+
ATP
+
30°C
P
P
LATENT
PHOSPHO-
TETRAMER
INTERMEDIATE
RATE-LIMITING
STAGE
PEPTIDE
+
0°C
30°C
P
P
P
P
ACTIVATED
PHOSPHO-TETRAMER
PEPTIDE-COMPLEX
ACTIVATED
PHOSPHO-TETRAMER

Fig.9.

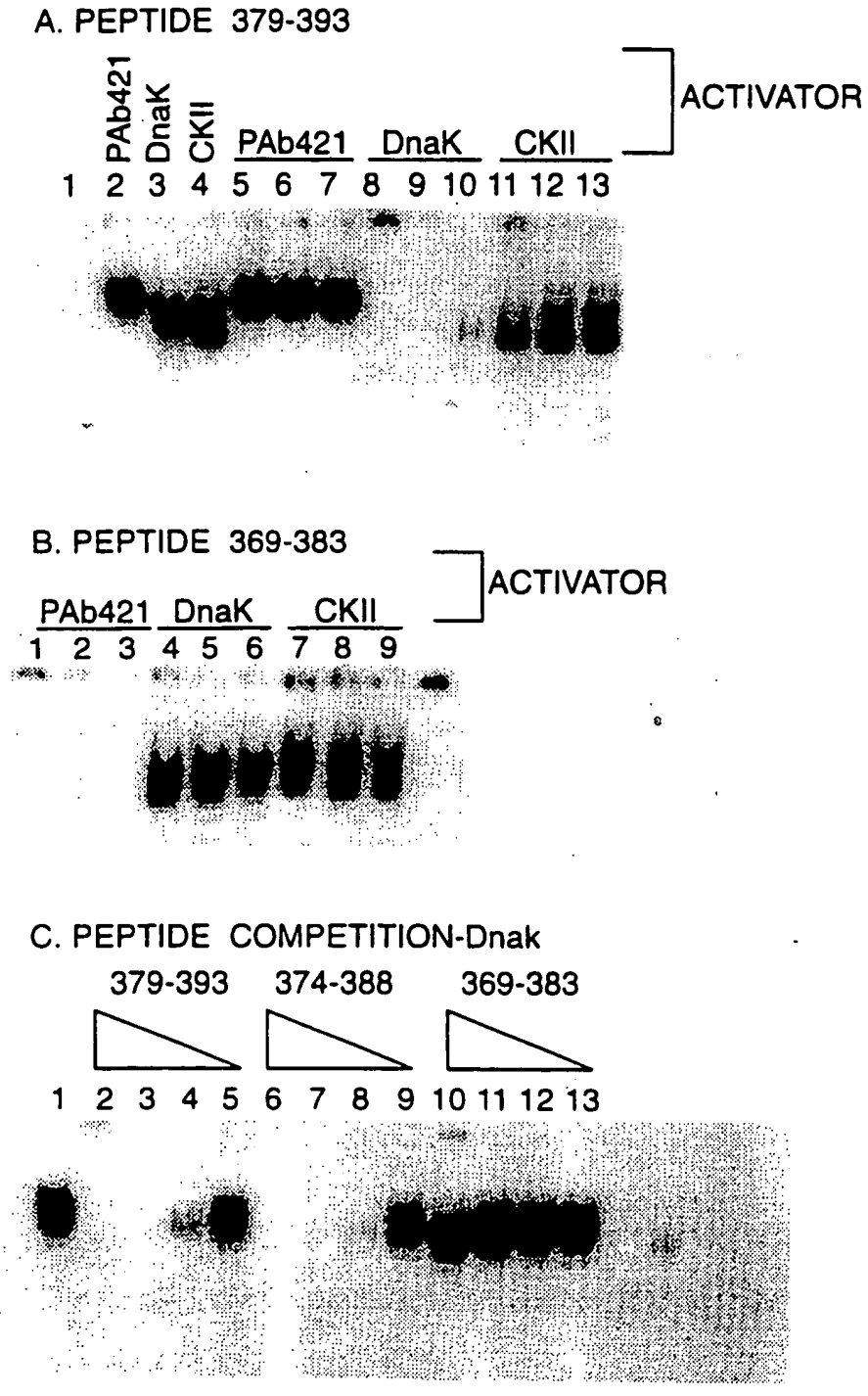

A. PEPTIDE 379-393
PAb421
DnaK
CKII
PAb421 DnaK CKII
ACTIVATOR
1 2 3 4 5 6 7 8 9 10 11 12 13
B. PEPTIDE 369-383
PAb421 DnaK CKII
ACTIVATOR
1 2 3 4 5 6 7 8 9
C. PEPTIDE COMPETITION-Dnak
379-393 374-388 369-383
1 2 3 4 5 6 7 8 9 10 11 12 13

Fig.10.

D:379-393 RHKKLMFKTEGPDSD
C:376-390 STSRHKKLMFKTEGP
B:371-385 SKKGQSTSRHKKLMF
A:366-380 SSHLKSKKGQSTSRH

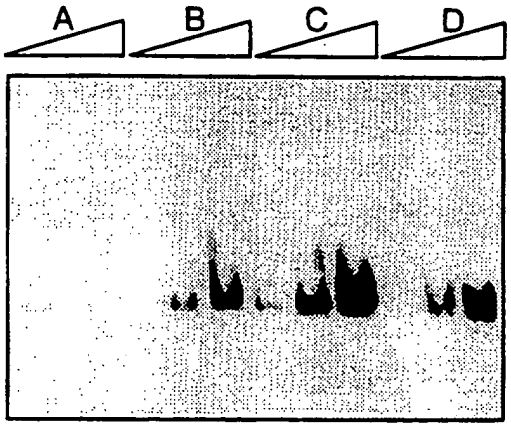


Fig.11.

D:379-393 RHKKLMFKTEGPDSD
C:376-390 STSRHKKLMFKTEGP
B:371-385 SKKGQSTSRHKKLMF
A:366-380 SSHLKSKKGQSTSRH

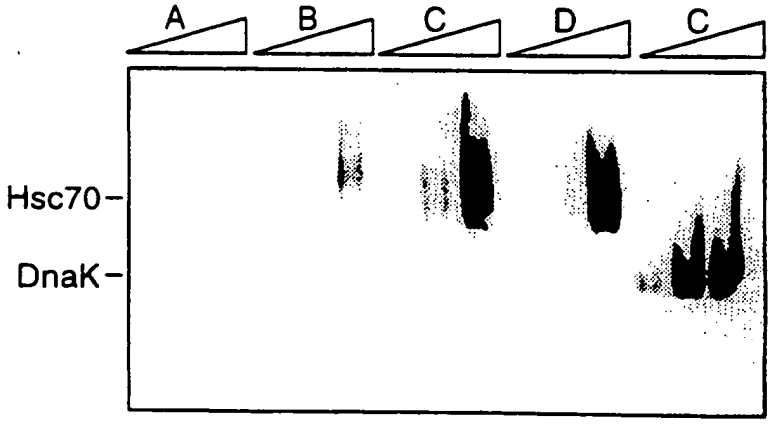


Fig.12.

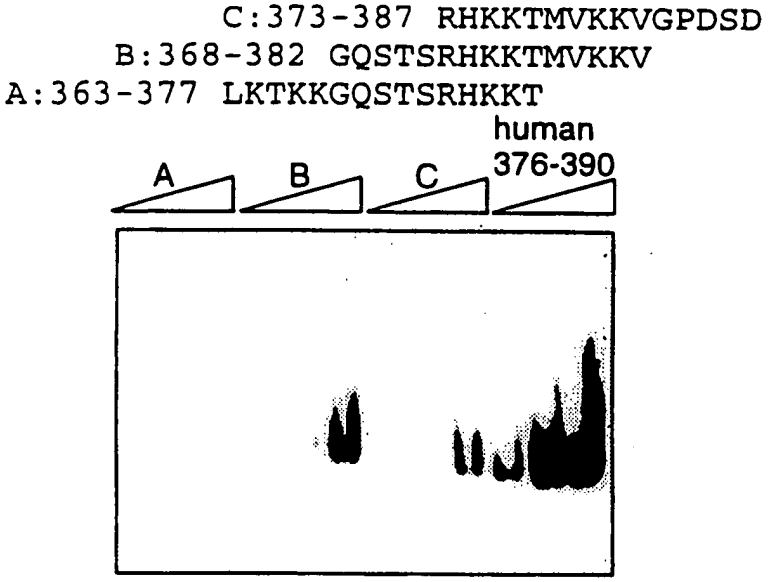

C:373-387 RHKKTMVKKVGPDSD
B:368-382 GQSTSRHKKTMVKKV
A:363-377 LKTKKGQSTSRHKKT
human
376-390
A
B
C

Fig.13.

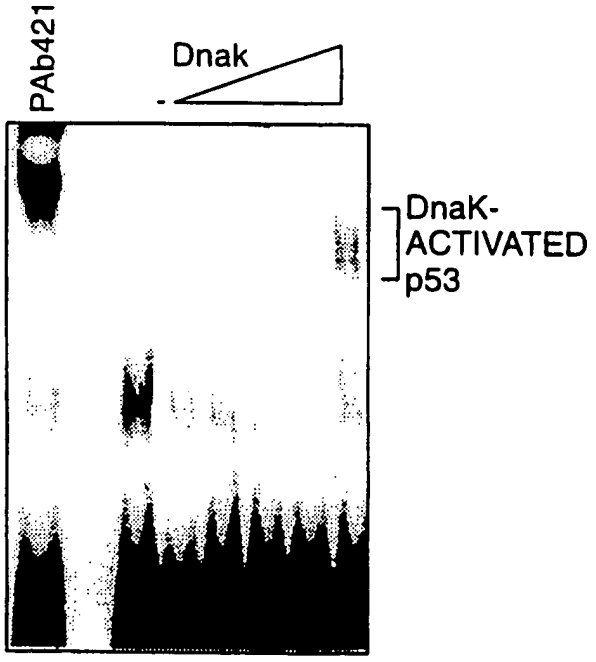
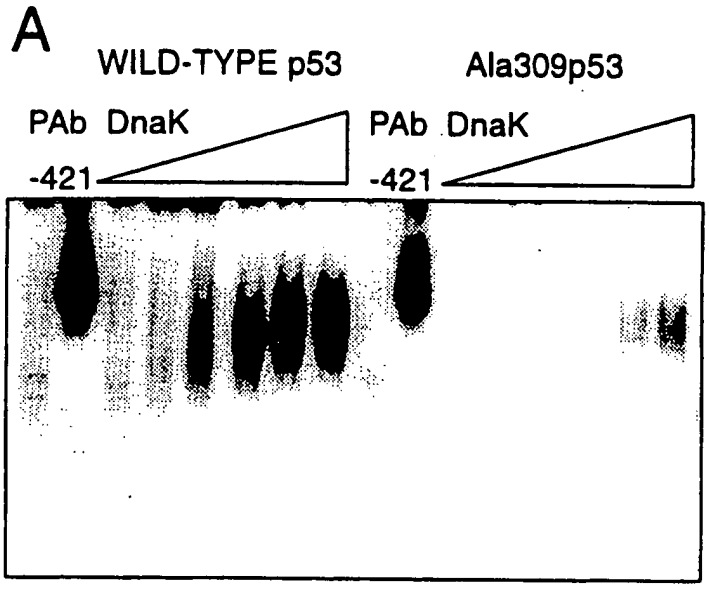

PAb421
Dnak
DnaK-
ACTIVATED
p53

Fig.14.


A
WILD-TYPE p53
Ala309p53
PAb DnaK
PAb DnaK
-421
-421

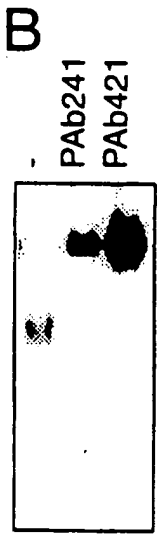

B
-
PAb241
PAb421

Fig.15.

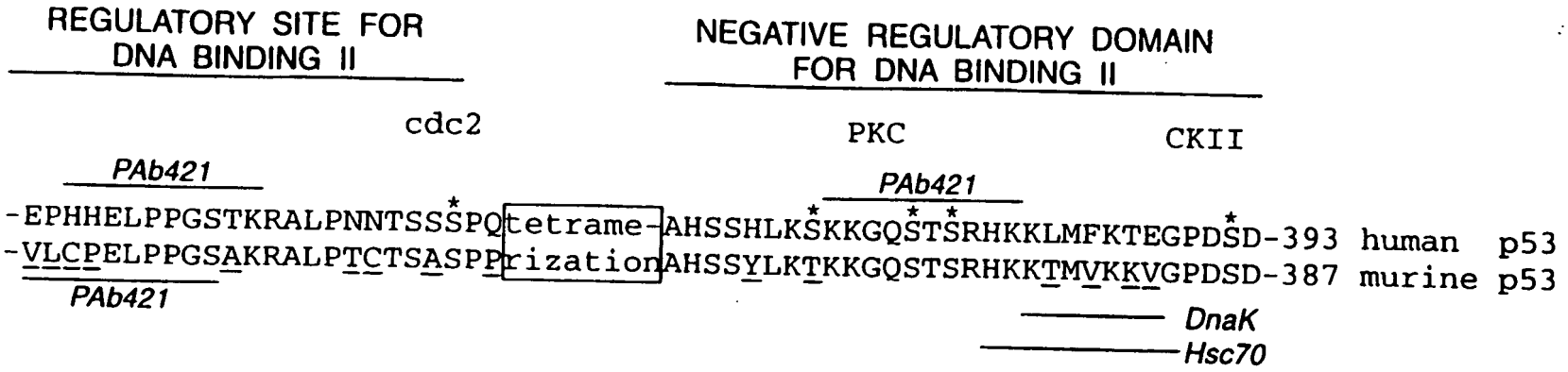


Fig.16.

REGULATORY DOMAIN I-SEQUENCES OF MOUSE AND HUMAN P53

HUMAN-367-SHLKSKKGQSTSRHKKLMFKTEGPDSD-393

MOUSE-361-SYLKTKKGQSTSRHKKTMVKKVGPDSD-387

DNAK BINDING SITE

REGULATORY DOMAIN II-SEQUENCES OF MOUSE AND HUMAN P53

HUMAN-293-GEPHHELPPGSTKRALPNNTSSSP-316

MOUSE-287-EVLCPELPPGSAKRALPTCTSASP-310

PAB241 BINDING SITE ON MOUSE P53

PCT/GB96/02605

SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/GB 96/02605

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C12N15/70 C07K14/82 C07K14/47 C07K14/245
G01N33/68 C07K19/00 A61K38/17 A61K38/04 A61K39/395
A61K38/46

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	CELL, vol. 83, 20 October 1995, pages 237-245, XP002003077 HUPP ET AL.: "Small peptides activate the latent sequence-specific DNA binding function of p53" see the whole document	1-5, 7-13, 17-19
A	---	14-16
P,X	WO 96 25434 A (THE WINSTAR INSTITUTE) 22 August 1996 see the whole document	1-3,8, 11-13, 15-18
A	---	4,7-10, 19
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 January 1997	07.02.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Gac, G

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INTERNATIONAL SEARCH REPORT

Intr onal Application No
PCT/GB 96/02605

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 06661 A (RESEARCH DEVELOPMENT FOUNDATION) 9 March 1995 see page 7 see page 19 see page 21, line 25 see page 22, line 30 - line 33 see page 23	1,8, 11-13
A	WO 94 12202 A (UNIVERSITY OF DUNDEE) 9 June 1994 cited in the application see whole document, especially pages 5-10, 31 and 32 see figure 5A	1,3,8-16
X	COLD SPRING HARBOR SYMP. QUANT. BIOL., vol. 60, 1995, pages 417-428, XP000613691 GETHING ET AL.: "Binding sites for HSP70 molecular chaperones in natural proteins" see page 417 - page 418	1,3,8, 11-13
A	see page 423 - page 425	14,17
A	COLD SPRING HARBOR SYMP. QUANT. BIOL., vol. 59, 1 - 8 June 1994, pages 195-206, XP000569570 HUPP ET AL.: "Regulation of the cryptic sequence-specific DNA-binding function of p53 by protein kinases" cited in the application see the whole document	1-3, 8-13, 15-19
A	J. BIOL. CHEM., vol. 270, no. 10, 10 March 1995, pages 5405-5411, XP002003076 TAKENADA ET AL.: "Regulation of the sequence-specific DNA binding function of p53 by protein kinase C and protein phosphatases" see the whole document	1-3,5, 8-13
A	CURRENT BIOLOGY, vol. 4, no. 10, 1 October 1994, pages 865-875, XP000569567 HUPP ET AL.: "Allosteric activation of latent p53 tetramers" see the whole document	1,2,8-13

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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/GB 96/02605

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NUCLEIC ACIDS RES., vol. 21, no. 14, 1993, pages 3167-3174, XP002022935 HUPP ET AL.: "Activation of the cryptic DNA binding function of mutant forms of p53" cited in the application see the whole document ---	1-3,8-14
A	EMBO J., vol. 11, no. 10, 1992, pages 3513-3520, XP002022936 HAINAUT ET AL.: "Interaction of heat-shock protein 70 with p53 translated in vitro : evidence for interaction with dimeric p53 and for a role in the regulation of p53 conformation" see the whole document ---	1-4,8-14
A	CELL, vol. 71, 27 November 1992, pages 875-886, XP002022937 HUPP ET AL.: "Regulation of the specific DNA binding function of p53" cited in the application see the whole document ---	1-3,8-14
A	PROC. NATL ACAD. SCI., vol. 92, June 1995, pages 5729-5733, XP002022938 BAYLE ET AL.: "The carboxy-terminal domain of the p53 protein regulates sequence-specific DNA binding through its nonspecific nucleic acid-binding activity" see the whole document ---	1-3,8-13
A	ONCOGENE, vol. 10, no. 6, 16 March 1995, pages 1167-1174, XP000569577 WOLKOVICZ ET AL.: "The DNA binding activity of wild-type p53 is modulated by blocking its various antigenic epitopes" see the whole document ---	1,2,8, 11,12

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INTERNATIONAL SEARCH REPORT

Int onal Application No
PCT/GB 96/02605

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE CANCERLIT AN: 95614994, (dialog AN= 1072690), XP002022939 see abstract & 3RD INT. CONF. MEDIT. SOC. TUMOR MARKER ONCOL., 26 - 29 October 1994, ATHENS (GR), page 118 HUPP: "Regulation of the DNA binding function and stability of human p53, rescuing wild-type function from mutant proteins" see abstract	1,8-13, 17
A	FR 2 662 698 A (CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE) 6 December 1991 cited in the application see whole document, especially page 8 & WO 91 18981 A	6

1

INTERNATIONAL SEARCH REPORT

information on patent family members

Inte ..nal Application No
PCT/GB 96/02605

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9625434	22-08-96	AU-A-	4915096	04-09-96
WO-A-9506661	09-03-95	AU-A-	7642694	22-03-95
		CA-A-	2170605	09-03-95
		CN-A-	1133595	16-10-96
		EP-A-	0716660	19-06-96
		ZA-A-	9406595	28-02-96
WO-A-9412202	09-06-94	AU-A-	5533194	22-06-94
		CA-A-	2150265	09-06-94
		EP-A-	0675729	11-10-95
		JP-T-	8505607	18-06-96
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		DE-D-	69122472	07-11-96
		EP-A-	0485578	20-05-92
		WO-A-	9118981	12-12-91
		JP-T-	5502885	20-05-93